POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH

RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Application number 60/454,300 filed March 12, 2003 and U.S. Provisional Application number 60/454,432 filed March 12, 2003. The entire teachings of the referenced Provisional Applications are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Angiogenesis, the development of new blood vessels from the endothelium of a preexisting vasculature, is a critical process in the growth, progression, and metastasis of solid tumors within the host. During physiologically normal angiogenesis, the autocrine, paracrine, and amphicrine interactions of the vascular endothelium with its surrounding stromal components are tightly regulated both spatially and temporally. Additionally, the levels and activities of proangiogenic and angiostatic cytokines and growth factors are maintained in balance. In contrast, the pathological angiogenesis necessary for active tumor growth is sustained and persistent, representing a dysregulation of the normal angiogenic system. Solid and hematopoietic tumor types are particularly associated with a high level of abnormal angiogenesis.

It is generally thought that the development of tumor consists of sequential, and interrelated steps that lead to the generation of an autonomous clone with aggressive growth potential. These steps include sustained growth and unlimited self-renewal. Cell populations in a tumor are generally characterized by growth signal self-sufficiency, decreased sensitivity to growth suppressive signals, and resistance to apoptosis. Genetic or cytogenetic events that initiate aberrant growth sustain cells in a prolonged "ready" state by preventing apoptosis.

It is a goal of the present disclosure to provide agents and therapeutic treatments for inhibiting angiogenesis and tumor growth.

SUMMARY OF THE INVENTION

In certain aspects, the disclosure provides polypeptide agents that inhibit EphB4 or EphrinB2 mediated functions, including monomeric ligand binding portions of the EphB4 and EphrinB2 proteins and antibodies that bind to and affect EphB4 or EphrinB2 in particular ways. As demonstrated herein, EphB4 and EphrinB2 participate in various disease states, including cancers and diseases related to unwanted or excessive angiogenesis. Accordingly, certain polypeptide agents disclosed herein may be used to treat such diseases. In further aspects, the disclosure relates to the discovery that EphB4 and/or EphrinB2 are expressed, often at high levels, in a variety of tumors. Therefore, polypeptide agents that downregulate EphB4 or EphrinB2 function may affect tumors by a direct effect on the tumor cells as well as an indirect effect on the angiogenic processes recruited by the tumor. In certain embodiments, the disclosure provides the identity of tumor types particularly suited to treatment with an agent that downregulates EphB4 or EphrinB2 function.

In certain aspects, the disclosure provides soluble EphB4 polypeptides comprising an amino acid sequence of an extracellular domain of an EphB4 protein. The soluble EphB4 polypeptides bind specifically to an EphrinB2 polypeptide. The term "soluble" is used merely to indicate that these polypeptides do not contain a transmembrane domain or a portion of a transmembrane domain sufficient to compromise the solubility of the polypeptide in a physiological salt solution. Soluble polypeptides are preferably prepared as monomers that compete with EphB4 for binding to ligand such as EphrinB2 and inhibit the signaling that results from EphB4 activation. Optionally, a soluble polypeptide may be prepared in a multimeric form, by, for example, expressing as an Fc fusion protein or fusion with another multimerization domain. Such multimeric forms may have complex activities, having agonistic or antagonistic effects depending on the context. In certain embodiments the soluble EphB4 polypeptide comprises a globular domain of an EphB4 protein. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-522 of the amino acid sequence defined by Figure 65. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-412 of the amino acid sequence defined by Figure 65. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-312 of the amino acid sequence defined by Figure 65. A soluble EphB4 polypeptide may comprise a sequence as set forth in Figure 1 or 2. In certain embodiments, the soluble EphB4 polypeptide may inhibit the interaction between

Ephrin B2 and EphB4. The soluble EphB4 polypeptide may inhibit clustering of or phosphorylation of Ephrin B2 or EphB4. Phosphorylation of EphrinB2 or EphB4 is generally considered to be one of the initial events in triggering intracellular signaling pathways regulated by these proteins. As noted above, the soluble EphB4 polypeptide may be prepared as a monomeric or multimeric fusion protein. The soluble polypeptide may include one or more modified amino acids. Such amino acids may contribute to desirable properties, such as increased resistance to protease digestion.

In certain aspects, the disclosure provides soluble EphrinB2 polypeptides comprising an amino acid sequence of an extracellular domain of an EphrinB2 protein. The soluble EphrinB2 polypeptides bind specifically to an EphB4 polypeptide. The term "soluble" is used merely to indicate that these polypeptides do not contain a transmembrane domain or a portion of a transmembrane domain sufficient to compromise the solubility of the polypeptide in a physiological salt solution. Soluble polypeptides are preferably prepared as monomers that compete with EphrinB2 for binding to ligand such as EphB4 and inhibit the signaling that results from EphrinB2 activation. Optionally, a soluble polypeptide may be prepared in a multimeric form, by, for example, expressing as an Fc fusion protein or fusion with another multimerization domain. Such multimeric forms may have complex activities, having agonistic or antagonistic effects depending on the context. A soluble EphrinB2 polypeptide may comprise residues 1-225 of the amino acid sequence defined by Figure 66. A soluble EphrinB2 polypeptide may comprise a sequence defined by Figure 3. In certain embodiments, the soluble EphrinB2 polypeptide may inhibit the interaction between Ephrin B2 and EphB4. The soluble EphrinB2 polypeptide may inhibit clustering of or phosphorylation of EphrinB2 or EphB4. As noted above, the soluble EphrinB2 polypeptide may be prepared as a monomeric or multimeric fusion protein. The soluble polypeptide may include one or more modified amino acids. Such amino acids may contribute to desirable properties, such as increased resistance to protease digestion.

In certain aspects, the disclosure provides antagonist antibodies for EphB4 and EphrinB2. An antibody may be designed to bind to an extracellular domain of an EphB4 protein and inhibit an activity of the EphB4. An antibody may be designed to bind to an extracellular domain of an Ephrin B2 protein and inhibit an activity of the Ephrin B2. An antibody may be designed to inhibit the interaction between Ephrin B2 and EphB4. An antagonist antibody will generally affect Eph and/or Ephrin signaling. For example, an antibody may inhibit clustering or

phosphorylation of Ephrin B2 or EphB4. An antagonist antibody may be essentially any polypeptide comprising a variable portion of an antibody, including, for example, monoclonal and polyclonal antibodies, single chain antibodies, diabodies, minibodies, etc.

In certain aspects, the disclosure provides pharmaceutical formulations comprising a polypeptide reagent and a pharmaceutically acceptable carrier. The polypeptide reagent may be any disclosed herein, including, for example, soluble EphB4 or EphrinB2 polypeptides and antagonist antibodies. Additional formulations include cosmetic compositions and diagnostic kits.

In certain aspects the disclosure provides methods of inhibiting signaling through Ephrin B2/EphB4 pathway in a cell. A method may comprise contacting the cell with an effective amount of a polypeptide agent, such as (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; or (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

In certain aspects the disclosure provides methods for reducing the growth rate of a tumor, comprising administering an amount of a polypeptide agent sufficient to reduce the growth rate of the tumor, wherein the polypeptide agent is selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2. Optionally, the tumor comprises cells expressing a higher level of EphB4 and/or EphrinB2 than noncancerous cells of a comparable tissue.

In certain aspects, the disclosure provides methods for treating a patient suffering from a cancer. A method may comprise administering to the patient a polypeptide agent selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2. Optionally, the cancer comprises cancer cells expressing EphrinB2 and/or EphB4 at a higher level than noncancerous cells of a comparable tissue. The cancer may be a metastatic cancer. The cancer may be selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia. Optionally, the cancer is an angiogenesis-dependent cancer or an angiogenesis independent cancer. The polypeptide agent employed may inhibit clustering or phosphorylation of Ephrin B2 or EphB4. A polypeptide agent may be co-administered with one or more additional anti-cancer chemotherapeutic agents that inhibit cancer cells in an additive or synergistic manner with the polypeptide agent.

In certain aspects, the disclosure provides methods of inhibiting angiogenesis. A method may comprise contacting a cell with an amount of a polypeptide agent sufficient to inhibit angiogenesis, wherein the polypeptide agent is selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

In certain aspects, the disclosure provides methods for treating a patient suffering from an angiogenesis-associated disease, comprising administering to the patient a polypeptide agent

selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2. The soluble polypeptide may be formulated with a pharmaceutically acceptable carrier. An angiogenesis related disease or unwanted angiogenesis related process may be selected from the group consisting of angiogenesis-dependent cancer, benign tumors, inflammatory disorders, chronic articular rheumatism and psoriasis, ocular angiogenic diseases, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, wound granulation, wound healing, telangiectasia psoriasis scleroderma, pyogenic granuloma, cororany collaterals, ischemic limb angiogenesis, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, and hematopoiesis. An polypeptide agent may be co-administered with at least one additional anti-angiogenesis agent that inhibits angiogenesis in an additive or synergistic manner with the soluble polypeptide.

In certain aspects, the disclosure provides for the use of a polypeptide agent in the manufacture of medicament for the treatment of cancer or an angiogenesis related disorder, wherein the polypeptide agent is selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

In certain aspects, the disclosure provides methods for for treating a patient suffering from a cancer, comprising: (a) identifying in the patient a tumor having a plurality of cancer cells that express EphB4 and/or EphrinB2; and (b) administering to the patient a polypeptide agent

selected from the group consisting of: (i) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (ii) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (iii) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (iv) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2. Optionally, a method may comprise identifying in the patient a tumor having a plurality of cancer cells having a gene amplification of the EphB4 and/or EphrinB2 gene.

In certain aspects, the disclosure provides methods for identifying a tumor that is suitable for treatment with an EphrinB2 or EphB4 antagonist. A method may comprise detecting in the tumor cell one or more of the following characteristics: (a) expression of EphB4 protein and/or mRNA; (b) expression of EphrinB2 protein and/or mRNA; (c) gene amplification of the EphB4 gene; or (d) gene amplification of the EphrinB2 gene. A tumor cell having one or more of characteristics (a)-(d) may be suitable for treatment with an EphrinB2 or EphB4 antagonist, such as a polypeptide agent described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows amino acid sequence of the B4ECv3 protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 2 shows amino acid sequence of the B4ECv3NT protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 3 shows amino acid sequence of the B2EC protein (predicted sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown).

Figure 4 shows amino acid sequence of the B4ECv3-FC protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 5 shows amino acid sequence of the B2EC-FC protein (predicted sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown).

Figure 6 shows B4EC-FC binding assay (Protein A-agarose based).

Figure 7 shows B4EC-FC inhibition assay (Inhibition in solution).

Figure 8 shows B2EC-FC binding assay (Protein-A-agarose based assay).

Figure 9 shows chemotaxis of HUAEC in response to B4Ecv3.

Figure 10 shows chemotaxis of HHEC in response to B2EC-FC.

Figure 11 shows chemotaxis of HHAEC in response to B2EC.

Figure 12 shows effect of B4Ecv3 on HUAEC tubule formation.

Figure 13 shows effect of B2EC-FC on HUAEC tubule formation.

Figure 14 is a schematic representation of human Ephrin B2 constructs.

Figure 15 is a schematic representation of human EphB4 constructs.

Figure 16 shows the domain structure of the recombinant soluble EphB4EC proteins. Designation of the domains are as follows: L - leader peptide, G – globular (ligand-binding domain), C – Cys-rich domain, F1, F2 – fibronectin type III repeats, H – 6 x His-tag.

Figure 17 shows purification and ligand binding properties of the EphB4EC proteins. **A.** SDS-PAAG gel electrophoresis of purified EphB4-derived recombinant soluble proteins (Coomassie-stained). **B.** Binding of Ephrin B2-AP fusion to EphB4-derived recombinant proteins immobilized on Ni-NTA-agarose beads. Results of three independent experiments are shown for each protein. Vertical axis – optical density at 420 nm.

Figure 18 shows that EphB4v3 inhibits chemotaxis.

Figure 19 shows that EphB4v3 inhibits tubule formation on Matrigel. **A** displays the strong inhibition of tubule formation by B4v3 in a representative experiment. **B** shows a quantitation of the reduction of tube-length obtained with B4v3 at increasing concentrations as well as a reduction in the number of junctions, in comparison to cells with no protein. Results are displayed as mean values _ S.D. obtained from three independent experiments performed with duplicate wells.

Figure 20 shows that soluble EphB4 has no detectable cytotoxic effect as assessed by MTS assay.

Figure 21 shows that B4v3 inhibits invasion and tubule formation by endothelial cells in the Matrigel assay. (*A*) to detect total invading cells, photographed at 20X magnification or with Masson's Trichrome Top left of A B displays section of a Matrigel plug with no GF , *top right* of *A* displays section with B4IgG containing GF and *lower left* section contains GF, and lower right shows GF in the presence of B4v3. Significant invasion of endothelial cells is only seenin GF containing Matrigel. *Top right* displays an area with a high number of invaded cells induced by B4IgG, which signifies the dimeric form of B4v3. The *left upper parts* of the pictures correspond to the cell layers formed around the Matrigel plug from which cells invade toward the center of the plug located in the direction of the *right lower corner.* Total cells in sections of the Matrigel plugs were quantitated with Scion Image software. Results obtained from two experiments with duplicate plugs are displayed as mean values _ S.D.

Figure 22 shows tyrosine phosphorylation of EphB4 receptor in PC3 cells in response to stimulation with EphrinB2-Fc fusion in presence or absence of EphB4-derived recombinant soluble proteins.

Figure 23 shows effects of soluble EphB4ECD on viability and cell cycle. A) 3-day cell viability assay of two HNSCC cell lines. B) FACS analysis of cell cycle in HNSCC-15 cells treated as in A. Treatment of these cells resulted in accumulation in subG0/G1 and S/G2 phases as indicated by the arrows.

Figure 24 shows that B4v3 inhibitis neovascular response in a murine corneal hydron micropocket assay.

Figure 25 shows that that SCC15, B16, and MCF-7 co-injected with sB4v3 in the presence of matrigel and growth factors, inhibits the in vivo tumor growth of these cells.

Figure 26 shows that soluble EphB4 causes apoptosis, necrosis and decreased angiogenesis in threee tumor types, B16 melanoma, SCC15, head and neck carcinoma, and MCF-7 Breast carcinoma. Tumors were injected premixed with Matrigel plus growth factors and soluble EphB4 subcutaneously. After 10 to 14 days, the mice were injected intravenously with fitc-lectin (green) to assess blood vessel perfusion. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic response Tumors treated with sEphB4 displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 27 shows expression of EphB4 in prostate cell lines. A) Western blot of total cell lysates of various prostate cancer cell lines, normal prostate gland derived cell line (MLC) and acute myeloblastic lymphoma cells (AML) probed with EphB4 monoclonal antibody. B) Phosphorylation of EphB4 in PC-3 cells determined by Western blot.

Figure 28 shows expression of EphB4 in prostate cancer tissue. Representative prostate cancer frozen section stained with EphB4 monoclonal antibody (top left) or isotype specific control (bottom left). Adjacent BPH tissue stained with EphB4 monoclonal antibody (top right). Positive signal is brown color in the tumor cells. Stroma and the normal epithelia are negative. Note membrane localization of stain in the tumor tissue, consistent with trans-membrane localization of EphB4. Representative QRT-PCR of RNA extracted from cancer specimens and adjacent BPH tissues (lower right).

Figure 29 shows downregulation of EphB4 in prostate cancer cells by tumor suppressors and RXR expression. A) PC3 cells were co-transfected with truncated CD4 and p53 or PTEN or vector only. 24 h later CD4-sorted cells were collected, lysed and analyzed sequentially by Western blot for the expression of EphB4 and β-actin, as a normalizer protein. B) Western blot as in (A) of various stable cell lines. LNCaP-FGF is a stable transfection clone of FGF-8, while CWR22R-RXR stably expresses the RXR receptor. BPH-1 was established from benign hypertrophic prostatic epithelium.

Figure 30 shows downregulation of EphB4 in prostate cancer cells by EGFR and IGFR-1. A) Western blot of PC3 cells treated with or without EGFR specific inhibitor AG1478 (1 nM) for 36 hours. Decreased EphB4 signal is observed after AG 1478 treatment. The membrane was stripped and reprobed with β-actin, which was unaffected. B) Western Blot of triplicate samples of PC3 cells treated with or without IGFR-1 specific neutralizing antibody MAB391 (2 μg/ml; overnight). The membrane was sequentially probed with EphB4, IGFR-1 and β-actin antibodies. IGFR-1 signal shows the expected repression of signal with MAB391 treatment.

Figure 31 shows effect of specific EphB4 AS-ODNs and siRNA on expression and prostate cell functions. A) 293 cells stably expressing full-length construct of EphB4 was used to evaluate the ability of siRNA 472 to inhibit EphB4 expression. Cells were transfected with 50 nM RNAi using Lipofectamine 2000. Western blot of cell lysates 40 h post transfection with control siRNA (green fluorescence protein; GFP siRNA) or EphB4 siRNA 472, probed with

EphB4 monoclonal antibody, stripped and reprobed with β-actin monoclonal antibody. B) Effect of EphB4 AS-10 on expression in 293 transiently expressing full-length EphB4. Cells were exposed to AS-10 or sense ODN for 6 hours and analyzed by Western blot as in (A). C) 48 h viability assay of PC3 cells treated with siRNA as described in the Methods section. Shown is mean ± s.e.m. of triplicate samples. D) 5-day viability assay of PC3 cells treated with ODNs as described in the Methods. Shown is mean ± s.e.m. of triplicate samples. E) Scrape assay of migration of PC3 cells in the presence of 50 nM siRNAs transfected as in (A). Shown are photomicrographs of representative 20x fields taken immediately after the scrape was made in the monolayer (0 h) and after 20h continued culture. A large number of cells have filled in the scrape after 20 h with control siRNA, but not with EphB4 siRNA 472. F) Shown is a similar assay for cells treated with AS-10 or sense ODN (both 10 μM). G) Matrigel invasion assay of PC3 cells transfected with siRNA or control siRNA as described in the methods. Cells migrating to the underside of the Matrigel coated insert in response to 5 mg/ml fibronectin in the lower chamber were fixed and stained with Giemsa. Shown are representative photomicrographs of control siRNA and siRNA 472 treated cells. Cell numbers were counted in 5 individual high-powered fields and the average ± s.e.m. is shown in the graph (bottom right).

Figure 32 shows effect of EphB4 siRNA 472 on cell cycle and apoptosis. A) PC3 cells transfected with siRNAs as indicated were analyzed 24 h post transfection for cell cycle status by flow cytometry as described in the Methods. Shown are the plots of cell number vs. propidium iodide fluorescence intensity. 7.9% of the cell population is apoptotic (in the Sub G0 peak) when treated with siRNA 472 compared to 1% with control siRNA. B) Apoptosis of PC3 cells detected by Cell Death Detection ELISA^{plus} kit as described in the Methods. Absorbance at 405 nm increases in proportion to the amount of histone and DNA-POD in the nuclei-free cell fraction. Shown is the mean ± s.e.m. of triplicate samples at the indicated concentrations of siRNA 472 and GFP siRNA (control).

Figure 33 shows that EphB4 and EphrinB2 are expressed in mesothelioma cell lines as shown by RT-PCR (A) and Western Blot (B).

Figure 34 shows expression of ephrin B2 and EphB4 by in situ hybridization in mesothelioma cells. NCI H28 mesothelioma cell lines cultured in chamber slides hybridized with

antisense probe to ephrin B2 or EphB4 (top row). Control for each hybridization was sense (bottom row). Positive reaction is dark blue cytoplasmic stain.

Figure 35 shows cellular expression of EphB4 and ephrin B2 in mesothelioma cultures. Immunofluorescence staining of primary cell isolate derived from pleural effusion of a patient with malignant mesothelioma and cell lines NCI H28, NCI H2373, and NCI H2052 for ephrin B2 and EphB4. Green color is positive signal for FITC labeled secondary antibody. Specificity of immunofluorescence staining was demonstrated by lack of signal with no primary antibody (first row). Cell nuclei were counterstained with DAPI (blue color) to reveal location of all cells. Shown are merged images of DAPI and FITC fluorescence. Original magnification 200X.

Figure 36 shows expression of ephrin B2 and EphB4 in mesothelioma tumor. Immunohistochemistry of malignant mesothelioma biopsy. H&E stained section to reveals tumor architecture; bottom left panel is background control with no primary antibody. EphB4 and ephrin B2 specific staining is brown color. Original magnification 200X.

Figure 37 shows effects of EPHB4 antisense probes (A) and EPHB4 siRNAs (B) on the growth of H28 cells.

Figure 38 shows effects of EPHB4 antisense probes (A) and EPHB4 siRNAs (B) on cell migration.

Figure 39 shows that EphB4 is expressed in HNSCC primary tissues and metastases. A) Top: Immunohistochemistry of a representative archival section stained with EphB4 monoclonal antibody as described in the methods and visualized with DAB (brown color) localized to tumor cells. Bottom: Hematoxylin and Eosin (H&E) stain of an adjacent section. Dense purple staining indicates the presence of tumor cells. The right hand column are frozen sections of lymph node metastasis stained with EphB4 polyclonal antibody (top right) and visualized with DAB. Control (middle) was incubation with goat serum and H&E (bottom) reveals the location of the metastatic foci surrounded by stroma which does not stain. B) In situ hybridization of serial frozen sections of a HNSCC case probed with EphB4 (left column) and ephrin B2 (right column) DIG labeled antisense or sense probes generated by run-off transcription. Hybridization signal (dark blue) was detected using alkaline-phosphatase-conjugated anti-DIG antibodies and sections were counterstained with Nuclear Fast Red. A serial section stained with H&E is shown (bottom left) to illustrate tumor architecture. C) Western blot of protein extract of patient samples

consisting of tumor (T), uninvolved normal tissue (N) and lymph node biopsies (LN). Samples were fractionated by polyacrylamide gel electrophoresis in 4-20% Tris-glycine gels and subsequently electroblotted onto nylon membranes. Membranes were sequentially probed with EphB4 monoclonal antibody and β-actin MoAb. Chemiluminescent signal was detected on autoradiography film. Shown is the EphB4 specific band which migrated at 120 kD and β-actin which migrated at 40 kD. The β-actin signal was used to control for loading and transfer of each sample.

Figure 40 shows that EphB4 is expressed in HNSCC cell lines and is regulated by EGF: A) Survey of EphB4 expression in SCC cell lines. Western blot of total cell lysates sequentially probed with EphB4 monoclonal antibody, stripped and reprobed with β-actin monoclonal antibody as described for Fig. 39C. B) Effect of the specific EGFR inhibitor AG1478 on EphB4 expression: Western blot of crude cell lysates of SCC15 treated with 0-1000 nM AG 1478 for 24 h in media supplemented with 10% FCS (left) or with 1 mM AG 1478 for 4, 8, 12 or 24 h (right). Shown are membranes sequentially probed for EphB4 and β-actin. C) Effect of inhibition of EGFR signaling on EphB4 expression in SCC cell lines: Cells maintained in growth media containing 10% FCS were treated for 24 hr with 1 μM AG 1478, after which crude cell lysates were analyzed by Western blots of cell lysates sequentially probed with for EGFR, EphB4, ephrin B2 and β-actin antibodies. Specific signal for EGFR was detected at 170 kD and ephrin B2 at 37 kD in addition to EphB4 and β-actin as described in Fig. 1C. β-actin serves as loading and transfer control.

Figure 41 shows mechanism of regulation of EphB4 by EGF: A) Schematic of the EGFR signaling pathways, showing in red the sites of action and names of specific kinase inhibitors used. B) SCC15 cells were serum-starved for 24 h prior to an additional 24 incubation as indicated with or without EGF (10 ng/ml), 3 μM U73122, or 5 μM SH-5, 5 μM SP600125, 25 nM LY294002, -- μM PD098095 or 5 μM SB203580. N/A indicates cultures that received equal volume of diluent (DMSO) only. Cell lysates were subjected to Western Blot with EphB4 monoclonal antibody. β-actin signal serves as control of protein loading and transfer.

Figure 42 shows that specific EphB4 siRNAs inhibit EphB4 expression, cell viability and cause cell cycle arrest. A) 293 cells stably expressing full length EphB4 were transfected with 50 nM RNAi using LipofectamineTM2000. 40 h post-transfection cells were harvested, lysed and

processed for Western blot. Membranes were probed with EphB4 monoclonal antibody, stripped and reprobed with β-actin monoclonal antibody as control for protein loading and transfer. Negative reagent control was RNAi to scrambled green fluorescence protein (GFP) sequence and control is transfection with LipofectamineTM2000 alone. B) MTT cell viability assays of SCC cell lines treated with siRNAs for 48 h as described in the Methods section. Shown is mean + s.e.m. of triplicate samples. C) SCC15 cells transfected with siRNAs as indicated were analyzed 24 h post transfection for cell cycle status by flow cytometry as described in the Methods. Shown are the plots of cell number vs. propidium iodide fluorescence intensity. Top and middle row show plots for cells 16 h after siRNA transfection, bottom row shows plots for cells 36 h post transfection. Specific siRNA and concentration are indicated for each plot. Lipo = LipofectamineTM200 mock transfection.

Figure 43 shows in vitro effects of specific EphB4 AS-ODNs on SCC cells. A) 293 cells transiently transfected with EphB4 full-length expression plasmid were treated 6 h post transfection with antisense ODNs as indicated. Cell lysates were collected 24 h after AS-ODN treatment and subjected to Western Blot. B) SCC25 cells were seeded on 48 well plates at equal densities and treated with EphB4 AS-ODNs at 1, 5, and 10 μM on days 2 and 4. Cell viability was measured by MTT assay on day 5. Shown is the mean + s.e.m. of triplicate samples. Note that AS-ODNs that were active in inhibiting EphB4 protein levels were also effective inhibitors of SCC15 cell viability. C) Cell cycle analysis of SCC15 cells treated for 36 h with AS-10 (bottom) compared to cells that were not treated (top). D) Confluent cultures of SCC15 cells scraped with a plastic Pasteur pipette to produce 3 mm wide breaks in the monolayer. The ability of the cells to migrate and close the wound in the presence of inhibiting EphB4 AS-ODN (AS-10) and non-inhibiting AS-ODN (AS-1) was assessed after 48 h. Scrambled ODN is included as a negative control ODN. Culture labeled no treatment was not exposed to ODN. At initiation of the experiment, all cultures showed scrapes of equal width and similar to that seen in 1 μM EphB4 AS-10 after 48 h. The red brackets indicate the width of the original scrape. E) Migration of SCC15 cells in response to 20 mg/ml EGF in two-chamber assay as described in the Methods. Shown are representative photomicrographs of non-treated (NT), AS-6 and AS-10 treated cells and 10 ng/ml Taxol as positive control of migration inhibition. F) Cell numbers were counted in 5 individual high-powered fields and the average + s.e.m. is shown in the graph.

Figure 44 shows that EphB4 AS-ODN inhibits tumor growth in vivo. Growth curves for SCC15 subcutaneous tumor xenografts in Balb/C nude mice treated with EphB4 AS-10 or scrambled ODN at 20 mg/kg/day starting the day following implantation of 5 x 106 cells. Control mice received and equal volume of diluent (PBS). Shown are the mean + s.e.m. of 6 mice/group. * P = 0.0001 by Student's t-test compared to scrambled ODN treated group.

Figure 45 shows that Ephrin B2, but not EphB4 is expressed in KS biopsy tissue. (A) In situ hybridization with antisense probes for ephrin B2 and EphB4 with corresponding H&E stained section to show tumor architecture. Dark blue color in the ISH indicates positive reaction for ephrin B2. No signal for EphB4 was detected in the Kaposi's sarcoma biopsy. For contrast, ISH signal for EphB4 is strong in squamous cell carcinoma tumor cells. Ephrin B2 was also detected in KS using EphB4-AP fusion protein (bottom left). (B) Detection of ephrin B2 with EphB4/Fc fusion protein. Adjacent sections were stained with H&E (left) to show tumor architecture, black rectangle indicates the area shown in the EphB4/Fc treated section (middle) detected with FITC-labeled anti-human Fc antibody as described in the methods section. As a control an adjacent section was treated with human Fc fragment (right). Specific signal arising from EphB4/Fc binding to the section is seen only in areas of tumor cells. (C) Co-expression of ephrin B2 and the HHV8 latency protein LANA1. Double-label confocal immunofluorescence microscopy with antibodies to ephrin B2 (red) LANA1 (green), or EphB4 (red) of frozen KS biopsy material directly demonstrates co-expression of LANA1 and ephrin B2 in KS biopsy. Coexpression is seen as yellow color. Double label confocal image of biopsy with antibodies to PECAM-1 (green) in cells with nuclear propidium iodide stain (red), demonstrating the vascular nature of the tumor.

Figure 46 shows that HHV-8 induces arterial marker expression in venous endothelial cells. (A) Immunofluorescence of cultures of HUVEC and HUVEC/BC-1 for artery/vein markers and viral proteins. Cultures were grown on chamber slides and processed for immunofluorescence detection of ephrin B2 (a, e, i), EphB4 (m, q, u), CD148 (j, v), and the HHV-8 proteins LANA1 (b, f, m) or ORF59 (r) as described in the Materials and Methods. Yellow color in the merged images of the same field demonstrate co-expression of ephrin B2 and LANA or ephrin B2 and CD148. The positions of viable cells were revealed by nuclear staining with DAPI (blue) in the third column (c, g, k, o, s, w). Photomicrographs are of representative fields. (B) RT-PCR of HUVEC and two HHV-8 infected cultures (HUVEC/BC-1 and

HUVEC/BC-3) for ephrin B2 and EphB4. Ephrin B2 product (200 bp) is seen in HUVEC/BC-1, HUVEC/BC-3 and EphB4 product (400 bp) is seen in HUVEC. Shown also is β-actin RT-PCR as a control for amount and integrity of input RNA.

Figure 47 shows that HHV-8 induces arterial marker expression in Kaposi's sarcoma cells. (A) Western blot for ephrin B2 on various cell lysates. SLK-vGPCR is a stable clone of SLK expressing the HHV-8 vGPCR, and SLK-pCEFL is control stable clone transfected with empty expression vector. SLK cells transfected with LANA or LANAΔ440 are SLK-LANA and SLK-Δ440 respectively. Quantity of protein loading and transfer was determined by reprobing the membranes with β-actin monoclonal antibody. (B) Transient transfection of KS-SLK cells with expression vector pvGPCR-CEFL resulted in the expression of ephrin B2 as shown by immunofluorescence staining with FITC (green), whereas the control vector pCEFL had no effect. KS-SLK cells (0.8 x 105/well) were transfected with 0.8 μg DNA using Lipofectamine 2000. 24 hr later cells were fixed and stained with ephrin B2 polyclonal antibody and FITC conjugated secondary antibody as described in the methods. (C) Transient transfection of HUVEC with vGPCR induces transcription from ephrin B2 luciferase constructs. 8 x 103 HUVEC in 24 well plates were transfected using Superfect with 0.8 μg/well ephrin B2 promoter constructs containing sequences from -2941 to -11 with respect to the translation start site, or two 5'-deletions as indicated, together with 80 ng/well pCEFL or pvGPCR-CEFL. Luciferase was determined 48 h post transfection and induction ratios are shown to the right of the graph. pGL3Basic is promoterless luciferase control vector. Luciferase was normalized to protein since GPCR induced expression of the cotransfected β-galactosidase. Graphed is mean + SEM of 6 replicates. Shown is one of three similar experiments.

Figure 48 shows that VEGF and VEGF-C regulate ephrin B2 expression. A) Inhibition of ephrin B2 by neutralizing antibodies. Cells were cultured in full growth medium and exposed to antibody (100 ng/ml) for 36 hr before collection and lysis for Western blot. B) For induction of ephrin B2 expression cells were cultured in EBM growth medium containing 5% serum lacking growth factors. Individual growth factors were added as indicated and the cells harvested after 36 h. Quantity of protein loading and transfer was determined by reprobing the membranes β-actin monoclonal antibody.

Figure 49 shows that Ephrin B2 knock-down with specific siRNA inhibits viability in KS cells and HUVEC grown in the presence of VEGF but not IGF, EGF or bFGF. A) KS-SLK cells were transfected with various siRNA to ephrin B2 and controls. After 48 hr the cells were harvested and crude cell lysates fractionated on 4-20% SDS-PAGE. Western blot was performed with monoclonal antibody to ephrin B2 generated in-house. The membrane was stripped and reprobed with β-actin monoclonal antibody (Sigma) to illustrate equivalent loading and transfer. B) 3 day cell viability assay of KS-SLK cultures in the presence of ephrin B2 and EphB4 siRNAs. 1 x 10^5 cells/well in 24-well plates were treated with 0, 10 and 100 ng/ml siRNAs as indicated on the graph. Viability of cultures was determined by MTT assay as described in the methods section. Shown are the mean + standard deviation of duplicate samples. C) HUVE cells were seeded on eight wells chamber slides coated with fibronectin. The HUVE cells were grown overnight in EGM-2 media, which contains all growth supplements. On the following day, the media was replaced with media containing VEGF (10ng/ml) or EGF, FGF and IGF as indicated. After 2 hrs of incubation at 37 °C, the cells were transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium containing 10 nM of siRNA to ephrin B2, Eph B4 or green fluorescence protein (GFP) as control. The cells were incubated for 2 hr and then the fresh media containing growth factors or VEGF alone was added to their respective wells. After 48 hrs, the cells were stained with crystal violet and the pictures were taken immediately by digital camera at 10X magnification.

Figure 50 shows that soluble EphB4 inhibits KS and EC cord formation and in vivo angiogenesis. Cord formation assay of HUVEC in MatrigelTM (upper row). Cells in exponential growth phase were treated overnight with the indicated concentrations of EphB4 extracellular domain (ECD) prior to plating on MatrigelTM. Cells were trypsinized and plated (1 x 10^5 cells/well) in a 24-well plate containing 0.5 ml MatrigelTM. Shown are representative 20X phase contrast fields of cord formation after 8 hr plating on MatrigelTM in the continued presence of the test compounds as shown. Original magnification 200 X. KS-SLK cells treated in a similar manner (middle row) in a cord formation assay on MatrigelTM. Bottom row shows in vivo MatrigelTM assay: MatrigelTM plugs containing growth factors and EphB4 ECD or PBS were implanted subcutaneously in the mid-ventral region of mice. After 7 days the plugs were removed, sectioned and stained with H&E to visualize cells migrating into the matrix. Intact

vessels with large lumens are observed in the control, whereas EphB4 ECD almost completely inhibited migration of cells into the Matrigel.

Figure 51 shows expression of EPHB4 in bladder cancer cell lines (A), and regulation of EPHB4 expression by EGFR signaling pathway (B).

Figure 52 shows that transfection of p53 inhibit the expression of EPHB4 in 5637 cell.

Figure 53 shows growth inhibition of bladder cancer cell line (5637) upon treatment with EPHB4 siRNA 472.

Figure 54 shows results on apoptosis study of 5637 cells transfected with EPHB4 siRNA 472.

Figure 55 shows effects of EPHB4 antisense probes on cell migration. 5637 cells were treated with EPHB4AS10 (10 μM).

Figure 56 shows effects of EPHB4 siRNA on cell invasion. 5637 cells were transfected with siRNA 472 or control siRNA.

Figure 57 shows comparison of EphB4 monoclonal antibodies by G250 and in pull-down assay.

Figure 58 shows that EphB4 antibodies inhibit the growth of SCC15 xenograft tumors.

Figure 59 shows that EphB4 antibodies cause apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

Figure 60 shows that systemic administration of EphB4 antibodies leads to tumor regression.

Figure 61 shows a genomic nucleotide sequence of human EphB4.

Figure 62 shows a cDNA nucleotide sequence of human EphB4.

Figure 63 shows a genomic nucleotide sequence of human Ephrin B2.

Figure 64 shows a cDNA nucleotide sequence of human Ephrin B2.

Figure 65 shows an amino acid sequence of human EphB4.

Figure 66 shows an amino acid sequence of human Ephrin B2.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

The current invention is based in part on the discovery that signaling through the ephrin/ephrin receptor pathway contributes to tumorigenesis. Applicants detected expression of ephrin B2 and EphB4 in tumor tissues and developed anti-tumor therapeutic agents for blocking signaling through the ephrin/ephrin receptor. In addition, the disclosure provides polypeptide therapeutic agents and methods for polypeptide-based inhibition of the function of EphB4 and/or Ephrin B2. Accordingly, in certain aspects, the disclosure provides numerous polypeptide compounds (agents) that may be used to treat cancer as well as angiogenesis related disorders and unwanted angiogenesis related processes.

As used herein, the terms Ephrin and Eph are used to refer, respectively, to ligands and receptors. They can be from any of a variety of animals (e.g., mammals/non-mammals, vertebrates/non-vertebrates, including humans). The nomenclature in this area has changed rapidly and the terminology used herein is that proposed as a result of work by the Eph Nomenclature Committee, which can be accessed, along with previously-used names at web site http://www.eph-nomenclature.com.

The work described herein, particularly in the examples, refers to Ephrin B2 and EphB4. However, the present invention contemplates any ephrin ligand and/or Eph receptor within their respective family, which is expressed in a tumor. The ephrins (ligands) are of two structural types, which can be further subdivided on the basis of sequence relationships and, functionally, on the basis of the preferential binding they exhibit for two corresponding receptor subgroups. Structurally, there are two types of ephrins: those which are membrane-anchored by a glycerophosphatidylinositol (GPI) linkage and those anchored through a transmembrane domain. Conventionally, the ligands are divided into the Ephrin-A subclass, which are GPI-linked proteins which bind preferentially to EphA receptors, and the Ephrin-B subclass, which are transmembrane proteins which generally bind preferentially to EphB receptors.

The Eph family receptors are a family of receptor protein-tyrosine kinases which are related to Eph, a receptor named for its expression in an erythropoietin-producing human hepatocellular carcinoma cell line. They are divided into two subgroups on the basis of the

relatedness of their extracellular domain sequences and their ability to bind preferentially to Ephrin-A proteins or Ephrin-B proteins. Receptors which interact preferentially with Ephrin-A proteins are EphA receptors and those which interact preferentially with Ephrin-B proteins are EphB receptors.

Eph receptors have an extracellular domain composed of the ligand-binding globular domain, a cysteine rich region followed by a pair of fibronectin type III repeats (e.g., see Figure 16). The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues; a protein tyrosine kinase domain; a sterile α-motif (SAM) and a PDZ-domain binding motif. EphB4 is specific for the membrane-bound ligand Ephrin B2 (Sakano, S. et al 1996; Brambilla R. et al 1995). Ephrin B2 belongs to the class of Eph ligands that have a transmembrane domain and cytoplasmic region with five conserved tyrosine residues and PDZ domain. Eph receptors are activated by binding of clustered, membrane attached ephrins (Davis S et al, 1994), indicating that contact between cells expressing the receptors and cells expressing the ligands is required for Eph activation.

Upon ligand binding, an Eph receptor dimerizes and autophosphorylate the juxtamembrane tyrosine residues to acquire full activation (Kalo MS et al, 1999, Binns KS, 2000). In addition to forward signaling through the Eph receptor, reverse signaling can occur through the ephrin Bs. Eph engagement of ephrins results in rapid phosphorylation of the conserved intracellular tyrosines (Bruckner K, 1997) and somewhat slower recruitment of PDZ binding proteins (Palmer A 2002). Recently, several studies have shown that high expression of Eph/ephrins may be associated with increased potentials for tumor growth, tumorigenicity, and metastasis (Easty DJ, 1999; Kiyokawa E, 1994; Tang XX, 1999; Vogt T, 1998; Liu W, 2002; Stephenson SA, 2001; Steube KG 1999; Berclaz G, 1996).

In certain embodiments, the present invention provides polypeptide therapeutic agents that inhibit activity of Ephrin B2, EphB4, or both. As used herein, the term "polypeptide therapeutic agent" or "polypeptide agent" is a generic term which includes any polypeptide that blocks signaling through the Ephrin B2/EphB4 pathway. A preferred polypeptide therapeutic agent of the invention is a soluble polypeptide of Ephrin B2 or EphB4. Another preferred polypeptide therapeutic agent of the invention is an antagonist antibody that binds to Ephrin B2 or EphB4. For example, such polypeptide therapeutic agent can inhibit function of Ephrin B2 or

EphB4, inhibit the interaction between Ephrin B2 and EphB4, inhibit the phosphorylation of Ephrin B2 or EphB4, or inhibit any of the downstream signaling events upon binding of Ephrin B2 to EphB4.

II. Soluble Polypeptides

In certain aspects, the invention relates to a soluble polypeptide comprising an extracellular domain of an Ephrin B2 protein (referred to herein as an Ephrin B2 soluble polypeptide) or comprising an extracellular domain of an EphB4 protein (referred to herein as an EphB4 soluble polypeptide). Preferably, the subject soluble polypeptide is a monomer and is capable of binding with high affinity to Ephrin B2 or EphB4. In a specific embodiment, the EphB4 soluble polypeptide of the invention comprises a globular domain of an EphB4 protein. Specific examples EphB4 soluble polypeptides are provided in Figures 1, 2, and 15. Specific examples of Ephrin B2 soluble polypeptides are provided in Figures 3 and 14.

As used herein, the subject soluble polypeptides include fragments, functional variants, and modified forms of EphB4 soluble polypeptide or an Ephrin B2 soluble polypeptide. These fragments, functional variants, and modified forms of the subject soluble polypeptides antagonize function of EphB4, Ephrin B2 or both.

In certain embodiments, isolated fragments of the subject soluble polypeptides can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding an EphB4 or Ephrin B2 soluble polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments that can function to inhibit function of EphB4 or Ephrin B2, for example, by testing the ability of the fragments to inhibit angiogenesis or tumor growth.

In certain embodiments, a functional variant of an EphB4 soluble polypeptide has an amino acid sequence that is at least 90%, 95%, 97%, 99% or 100% identical to residues 1-522, residues 1-412, or residues 1-312 of the amino acid sequence defined by Figure 65. In other embodiments, a functional variant of an Ephrin B2 soluble polypeptide has a sequence at least 90%, 95%, 97%, 99% or 100% identical to residues 1-225 of the amino acid sequence defined by Figure 66.

In certain embodiments, the present invention contemplates making functional variants by modifying the structure of the subject soluble polypeptide for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified soluble polypeptide are considered functional equivalents of the naturally-occurring EphB4 or Ephrin B2 soluble polypeptide. Modified soluble polypeptides can be produced, for instance, by amino acid substitution, deletion, or addition. For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains.

This invention further contemplates a method of generating sets of combinatorial mutants of the EphB4 or Ephrin B2 soluble polypeptides, as well as truncation mutants, and is especially useful for identifying functional variant sequences. The purpose of screening such combinatorial libraries may be to generate, for example, soluble polypeptide variants which can act as antagonists of EphB4, EphB2, or both. Combinatorially-derived variants can be generated which have a selective potency relative to a naturally occurring soluble polypeptide. Such variant proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding wild-type soluble polypeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of the protein of interest (e.g., a soluble polypeptide). Such variants, and the genes which encode them, can be utilized to alter the subject soluble polypeptide levels by modulating their half-life. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant soluble polypeptide levels within the cell. As above, such proteins, and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be

carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential soluble polypeptide sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al., (1981) Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acid Res. 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, soluble polypeptide variants (e.g., the antagonist forms) can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) Biochemistry 33:1565-1572; Wang et al., (1994) J. Biol. Chem. 269:3095-3099; Balint et al., (1993) Gene 137:109-118; Grodberg et al., (1993) Eur. J. Biochem. 218:597-601; Nagashima et al., (1993) J. Biol. Chem. 268:2888-2892; Lowman et al., (1991) Biochemistry 30:10832-10838; and Cunningham et al., (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by saturation mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of the subject soluble polypeptide.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of the subject soluble polypeptides. The most widely used techniques for screening

large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In certain embodiments, the subject soluble polypeptides of the invention include a a small molecule such as a peptide and a peptidomimetic. As used herein, the term "peptidomimetic" includes chemically modified peptides and peptide-like molecules that contain non-naturally occurring amino acids, peptoids, and the like. Peptidomimetics provide various advantages over a peptide, including enhanced stability when administered to a subject. Methods for identifying a peptidomimetic are well known in the art and include the screening of databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known crystal structures (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). Where no crystal structure of a target molecule is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems; San Leandro Calif.), contains about 100,000 compounds that are commercially available and also can be searched to identify potential peptidomimetics of the EphB4 or Ephrin B2 soluble polypeptides.

To illustrate, by employing scanning mutagenesis to map the amino acid residues of a soluble polypeptidewhich are involved in binding to another protein, peptidomimetic compounds can be generated which mimic those residues involved in binding. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al., (1986) J. Med. Chem. 29:295; and Ewenson et al., in Peptides: Structure and Function (Proceedings of the

9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al., (1985) Tetrahedron Lett 26:647; and Sato et al., (1986) J Chem Soc Perkin Trans 1:1231), and b-aminoalcohols (Gordon et al., (1985) Biochem Biophys Res Commun 126:419; and Dann et al., (1986) Biochem Biophys Res Commun 134:71).

In certain embodiments, the soluble polypeptides of the invention may further comprise post-translational modifications. Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. As a result, the modified soluble polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of a soluble polypeptide may be tested for its antagozing role in EphB4 or Ephrin B2 function, e.g, it inhibitory effect on angiogenesis or on tumor growth.

In certain aspects, functional variants or modified forms of the subject soluble polypeptides include fusion proteins having at least a portion of the soluble polypeptide and one or more fusion domains. Well known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, and an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), which are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt- conjugated resins are used. Another fusion domain well known in the art is green fluorescent protein (GFP). Fusion domains also include "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain embodiments, the soluble polypeptides of the present invention contain one or more modifications that are capable of stabilizing the soluble polypeptides. For example, such modifications enhance the in vitro half life of the soluble polypeptides, enhance circulatory half life of the soluble polypeptides or reducing proteolytic degradation of the soluble polypeptides.

In certain embodiments, soluble polypeptides (unmodified or modified) of the invention can be produced by a variety of art-known techniques. For example, such soluble polypeptides can be synthesized using standard protein chemistry techniques such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant G. A. (ed.), Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992). In addition, automated peptide synthesizers are commercially available (e.g., Advanced ChemTech Model 396; Milligen/Biosearch 9600). Alternatively, the soluble polypeptides, fragments or variants thereof may be recombinantly produced using various expression systems as is well known in the art (also see below).

III. Nucleic acids encoding soluble polypeptides

In certain aspects, the invention relates to isolated and/or recombinant nucleic acids encoding an EphB4 or Ephrin B2 soluble polypeptide. The subject nucleic acids may be single-stranded or double-stranded, DNA or RNA molecules. These nucleic acids are useful as therapeutic agents. For example, these nucleic acids are useful in making recombinant soluble polypeptides which are administered to a cell or an individual as therapeutics. Alternative, these nucleic acids can be directly administered to a cell or an individual as therapeutics such as in gene therapy.

In certain embodiments, the invention provides isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to a region of the nucleotide sequence depicted in Figure 62 or 63. One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to the subject nucleic acids, and variants of the subject nucleic acids are also within the scope of this invention. In further embodiments, the nucleic acid sequences of the invention can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

In other embodiments, nucleic acids of the invention also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequence depicted in Figure 62 or 63, or complement sequences thereof. As discussed above, one of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform

the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the subject nucleic acids due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

In certain embodiments, the recombinant nucleic acids of the invention may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate for a host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An

expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

In certain aspect of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding an EphB4 or Ephrin B2 soluble polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the soluble polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology*: *Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding a soluble polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

This invention also pertains to a host cell transfected with a recombinant gene including a coding sequence for one or more of the subject soluble polypeptide. The host cell may be any prokaryotic or eukaryotic cell. For example, a soluble polypeptide of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression

system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject soluble polypeptides. For example, a host cell transfected with an expression vector encoding an EphB4 soluble polypeptide can be cultured under appropriate conditions to allow expression of the EphB4 soluble polypeptide to occur. The EphB4 soluble polypeptide may be secreted and isolated from a mixture of cells and medium containing the soluble polypeptides. Alternatively, the soluble polypeptides may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The soluble polypeptides can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the soluble polypeptides. In a preferred embodiment, the soluble polypeptide is a fusion protein containing a domain which facilitates its purification.

A recombinant nucleic acid of the invention can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant soluble polypeptide include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

The preferred mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus

(pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant SLC5A8 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

IV. Antibodies

In certain aspects, the the present invention provides antagonist antibodies against Ephrin B2 or EphB4. As described herein, the term "antagonist antibody" refers to an antibody that inhibits function of Ephrin B2 or EphB4. Preferably, the antagonist antibody binds to an extracellular domain of Ephrin B2 or EphB4. It is understood that antibodies of the invention

may be polyclonal or monoclonal; intact or truncated, e.g., F(ab')2, Fab, Fv; xenogeneic, allogeneic, syngeneic, or modified forms thereof, e.g., humanized, chimeric, etc.

For example, by using immunogens derived from an Ephrin B2 or EphB4 polypeptide, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (see, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide. (e.g., a polypeptide or an antigenic fragment which is capable of eliciting an antibody response, or a fusion protein). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an Ephrin B2 or EphB4 polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In one embodiment, antibodies of the invention are specific for the extracellular portion of the Ephrin B2 or EphB4 protein. In another embodiment, antibodies of the invention are specific for the intracellular portion or the transmembrane portion of the Ephrin B2 or EphB4 protein. In a further embodiment, antibodies of the invention are specific for the extracellular portion of the Ephrin B2 or EphB4 protein.

Following immunization of an animal with an antigenic preparation of an Ephrin B2 or EphB4 polypeptide, antisera can be obtained and, if desired, polyclonal antibodies can be isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an Ephrin B2 or EphB4 polypeptide and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with an Ephrin B2 or EphB4 polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab)2 fragments can be generated by treating antibody with pepsin. The resulting F(ab)2 fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for an Ephrin B2 or EphB4 polypeptide conferred by at least one CDR region of the antibody. Techniques for the production of single chain antibodies (US Patent No. 4,946,778) can also be adapted to produce single chain antibodies. Also, transgenic mice or other organisms including other mammals, may be used to express humanized antibodies. In preferred embodiments, the antibodies further comprise a label attached thereto and able to be detected (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor).

In certain preferred embodiments, an antibody of the invention is a monoclonal antibody, and in certain embodiments the invention makes available methods for generating novel antibodies. For example, a method for generating a monoclonal antibody that binds specifically to an Ephrin B2 or EphB4 polypeptide may comprise administering to a mouse an amount of an immunogenic composition comprising the Ephrin B2 or EphB4 polypeptide effective to stimulate a detectable immune response, obtaining antibody-producing cells (e.g., cells from the spleen) from the mouse and fusing the antibody–producing cells with myeloma cells to obtain antibody-producing hybridomas, and testing the antibody-producing hybridomas to identify a hybridoma that produces a monocolonal antibody that binds specifically to the Ephrin B2 or EphB4 polypeptide. Once obtained, a hybridoma can be propagated in a cell culture, optionally in culture conditions where the hybridoma-derived cells produce the monoclonal antibody that binds specifically to the Ephrin B2 or EphB4 polypeptide. The monoclonal antibody may be purified from the cell culture.

In addition, the techniques used to screen antibodies in order to identify a desirable antibody may influence the properties of the antibody obtained. For example, an antibody to be used for certain therapeutic purposes will preferably be able to target a particular cell type. Accordingly, to obtain antibodies of this type, it may be desirable to screen for antibodies that bind to cells that express the antigen of interest (e.g., by fluorescence activated cell sorting).

Likewise, if an antibody is to be used for binding an antigen in solution, it may be desirable to test solution binding. A variety of different techniques are available for testing antibody:antigen interactions to identify particularly desirable antibodies. Such techniques include ELISAs, surface plasmon resonance binding assays (e.g. the Biacore binding assay, Bia-core AB, Uppsala, Sweden), sandwich assays (e.g. the paramagnetic bead system of IGEN International, Inc., Gaithersburg, Maryland), western blots, immunoprecipitation assays and immunohistochemistry.

V. Drug Screening Assays

There are numerous approaches to screening for polypeptide therapeutic agents as antagonists of EphB4, Ephrin B2 or both. For example, high-throughput screening of compounds or molecules can be carried out to identify agents or drugs which inhibit angiogenesis or inhibit tumor growth. Test agents can be any chemical (element, molecule, compound, drug), made synthetically, made by recombinant techniques or isolated from a natural source. For example, test agents can be peptides, polypeptides, peptoids, sugars, hormones, or nucleic acid molecules. In addition, test agents can be small molecules or molecules of greater complexity made by combinatorial chemistry, for example, and compiled into libraries. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic compounds. Test agents can also be natural or genetically engineered products isolated from lysates or growth media of cells -- bacterial, animal or plant -- or can be the cell lysates or growth media themselves. Presentation of test compounds to the test system can be in either an isolated form or as mixtures of compounds, especially in initial screening steps.

For example, an assay can be carried out to screen for compounds that specifically inhibit binding of Ephrin B2 (ligand) to EphB4 (receptor), or vice-versa, e.g., by inhibition of binding of labeled ligand- or receptor-Fc fusion proteins to immortalized cells. Compounds identified through this screening can then be tested in animals to assess their anti-angiogenesis or anti-tumor activity in vivo.

In one embodiment of an assay to identify a substance that interferes with interaction of two cell surface molecules (e.g., Ephrin B2 and EphB4), samples of cells expressing one type of

cell surface molecule (e.g., EphB4) are contacted with either labeled ligand (e.g., Ephrin B2, or a soluble portion thereof, or a fusion protein such as a fusion of the extracellular domain and the Fc domain of IgG) or labeled ligand plus a test compound (or group of test compounds). The amount of labeled ligand which has bound to the cells is determined. A lesser amount of label (where the label can be, for example, a radioactive isotope, a fluorescent or colormetric label) in the sample contacted with the test compound(s) is an indication that the test compound(s) interferes with binding. The reciprocal assay using cells expressing a ligand (e.g., an Ephrin B2 ligand or a soluble form thereof) can be used to test for a substance that interferes with the binding of an Eph receptor or soluble portion thereof.

An assay to identify a substance which interferes with interaction between an Eph receptor and an ephrin can be performed with the component (e.g., cells, purified protein, including fusion proteins and portions having binding activity) which is not to be in competition with a test compound, linked to a solid support. The solid support can be any suitable solid phase or matrix, such as a bead, the wall of a plate or other suitable surface (e.g., a well of a microtiter plate), column pore glass (CPG) or a pin that can be submerged into a solution, such as in a well. Linkage of cells or purified protein to the solid support can be either direct or through one or more linker molecules.

In one embodiment, an isolated or purified protein (e.g., an Eph receptor or an ephrin) can be immobilized on a suitable affinity matrix by standard techniques, such as chemical cross-linking, or via an antibody raised against the isolated or purified protein, and bound to a solid support. The matrix can be packed in a column or other suitable container and is contacted with one or more compounds (e.g., a mixture) to be tested under conditions suitable for binding of the compound to the protein. For example, a solution containing compounds can be made to flow through the matrix. The matrix can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds. Compounds which remain bound can be released by a suitable elution buffer. For example, a change in the ionic strength or pH of the elution buffer can lead to a release of compounds. Alternatively, the elution buffer can comprise a release component or components designed to disrupt binding of compounds (e.g., one or more ligands or receptors, as appropriate, or analogs thereof which can disrupt binding or competitively inhibit binding of test compound to the protein).

Fusion proteins comprising all, or a portion of, a protein (e.g., an Eph receptor or an ephrin) linked to a second moiety not occurring in that protein as found in nature can be prepared for use in another embodiment of the method. Suitable fusion proteins for this purpose include those in which the second moiety comprises an affinity ligand (e.g., an enzyme, antigen, epitope). The fusion proteins can be produced by inserting the protein (e.g., an Eph receptor or an ephrin) or a portion thereof into a suitable expression vector which encodes an affinity ligand. The expression vector can be introduced into a suitable host cell for expression. Host cells are disrupted and the cell material, containing fusion protein, can be bound to a suitable affinity matrix by contacting the cell material with an affinity matrix under conditions sufficient for binding of the affinity ligand portion of the fusion protein to the affinity matrix.

In one aspect of this embodiment, a fusion protein can be immobilized on a suitable affinity matrix under conditions sufficient to bind the affinity ligand portion of the fusion protein to the matrix, and is contacted with one or more compounds (e.g., a mixture) to be tested, under conditions suitable for binding of compounds to the receptor or ligand protein portion of the bound fusion protein. Next, the affinity matrix with bound fusion protein can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds without significantly disrupting binding of specifically bound compounds. Compounds which remain bound can be released by contacting the affinity matrix having fusion protein bound thereto with a suitable elution buffer (a compound elution buffer). In this aspect, compound elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the compound(s) tested to the receptor or ligand protein portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of compounds, or the elution buffer can comprise a release component or components designed to disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein (e.g., one or more ligands or receptors or analogs thereof which can disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein). Immobilization can be performed prior to, simultaneous with, or after contacting the fusion protein with compound, as appropriate. Various permutations of the method are possible, depending upon factors such as the compounds tested, the affinity matrix selected, and elution buffer formulation. For example, after the wash step, fusion protein with compound bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution

buffer). Where the fusion protein comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with compound bound thereto. Bound compound can then be released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

VI. Methods of Treatment

In certain embodiments, the present invention provides methods of inhibiting angiogenesis and methods of treating angiogenesis-associated diseases. In other embodiments, the present invention provides methods of inhibiting or reducing tumor growth and methods of treating an individual suffering from cancer. These methods involve administering to the individual a therapeutically effective amount of one or more polypeptide therapeutic agents as described above. These methods are particularly aimed at therapeutic and prophylactic treatments of animals, and more particularly, humans.

As described herein, angiogenesis-associated diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; inflammatory disorders such as immune and non-immune inflammation; chronic articular rheumatism and psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation and wound healing; telangiectasia psoriasis scleroderma, pyogenic granuloma, cororany collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, hematopoiesis.

It is understood that methods and compositions of the invention are also useful for treating any angiogenesis-independent cancers (tumors). As used herein, the term "angiogenesis-independent cancer" refers to a cancer (tumor) where there is no or little neovascularization in the tumor tissue.

In particular, polypeptide therapeutic agents of the present invention are useful for treating or preventing a cancer (tumor), including, but not limited to, colon carcinoma, breast cancer, mesothelioma, prostate cancer, bladder cancer, squamous cell carcinoma of the head and neck (HNSCC), Kaposi sarcoma, and leukemia.

In certain embodiments of such methods, one or more polypeptide therapeutic agents can be administered, together (simultaneously) or at different times (sequentially). In addition, polypeptide therapeutic agents can be administered with another type of compounds for treating cancer or for inhibiting angiogenesis.

In certain embodiments, the subject methods of the invention can be used alone. Alternatively, the subject methods may be used in combination with other conventional anti-cancer therapeutic approaches directed to treatment or prevention of proliferative disorders (e.g., tumor). For example, such methods can be used in prophylactic cancer prevention, prevention of cancer recurrence and metastases after surgery, and as an adjuvant of other conventional cancer therapy. The present invention recognizes that the effectiveness of conventional cancer therapies (e.g., chemotherapy, radiation therapy, phototherapy, immunotherapy, and surgery) can be enhanced through the use of a subject polypeptide therapeutic agent.

A wide array of conventional compounds have been shown to have anti-neoplastic activities. These compounds have been used as pharmaceutical agents in chemotherapy to shrink solid tumors, prevent metastases and further growth, or decrease the number of malignant cells in leukemic or bone marrow malignancies. Although chemotherapy has been effective in treating various types of malignancies, many anti-neoplastic compounds induce undesirable side effects. It has been shown that when two or more different treatments are combined, the treatments may work synergistically and allow reduction of dosage of each of the treatments, thereby reducing the detrimental side effects exerted by each compound at higher dosages. In other instances, malignancies that are refractory to a treatment may respond to a combination therapy of two or more different treatments.

When a polypeptide therapeutic agent of the present invention is administered in combination with another conventional anti-neoplastic agent, either concomitantly or sequentially, such therapeutic agent is shown to enhance the therapeutic effect of the anti-neoplastic agent or overcome cellular resistance to such anti-neoplastic agent. This allows

decrease of dosage of an anti-neoplastic agent, thereby reducing the undesirable side effects, or restores the effectiveness of an anti-neoplastic agent in resistant cells.

Pharmaceutical compounds that may be used for combinatory anti-tumor therapy include, merely to illustrate: aminoglutethimide, amsacrine, anastrozole, asparaginase, bcg, bicalutamide, bleomycin, buserelin, busulfan, campothecin, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, colchicine, cyclophosphamide, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, dienestrol, diethylstilbestrol, docetaxel, doxorubicin, epirubicin, estradiol, estramustine, etoposide, exemestane, filgrastim, fludarabine, fludrocortisone, fluorouracil, fluoxymesterone, flutamide, gemcitabine, genistein, goserelin, hydroxyurea, idarubicin, ifosfamide, imatinib, interferon, irinotecan, ironotecan, letrozole, leucovorin, leuprolide, levamisole, lomustine, mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine, mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, nocodazole, octreotide, oxaliplatin, paclitaxel, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, suramin, tamoxifen, temozolomide, teniposide, testosterone, thioguanine, thiotepa, titanocene dichloride, topotecan, trastuzumab, tretinoin, vinblastine, vincristine, vindesine, and vinorelbine.

These chemotherapeutic anti-tumor compounds may be categorized by their mechanism of action into, for example, following groups: anti-metabolites/anti-cancer agents, such as pyrimidine analogs (5-fluorouracil, floxuridine, capecitabine, gemcitabine and cytarabine) and purine analogs, folate antagonists and related inhibitors (mercaptopurine, thioguanine, pentostatin and 2-chlorodeoxyadenosine (cladribine)); antiproliferative/antimitotic agents including natural products such as vinca alkaloids (vinblastine, vincristine, and vinorelbine), microtubule disruptors such as taxane (paclitaxel, docetaxel), vincristin, vinblastin, nocodazole, epothilones and navelbine, epidipodophyllotoxins (etoposide, teniposide), DNA damaging agents (actinomycin, amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, doxorubicin, epirubicin, hexamethylmelamineoxaliplatin, iphosphamide, melphalan, merchlorehtamine, mitomycin, mitoxantrone, nitrosourea, plicamycin, procarbazine, taxol, taxotere, teniposide, triethylenethiophosphoramide and etoposide (VP16)); antibiotics such as dactinomycin (actinomycin D), daunorubicin, doxorubicin (adriamycin), idarubicin, anthracyclines,

mitoxantrone, bleomycins, plicamycin (mithramycin) and mitomycin; enzymes (L-asparaginase which systemically metabolizes L-asparagine and deprives cells which do not have the capacity to synthesize their own asparagine); antiplatelet agents; antiproliferative/antimitotic alkylating agents such as nitrogen mustards (mechlorethamine, cyclophosphamide and analogs, melphalan, chlorambucil), ethylenimines and methylmelamines (hexamethylmelamine and thiotepa), alkyl sulfonates-busulfan, nitrosoureas (carmustine (BCNU) and analogs, streptozocin), trazenes - dacarbazinine (DTIC); antiproliferative/antimitotic antimetabolites such as folic acid analogs (methotrexate); platinum coordination complexes (cisplatin, carboplatin), procarbazine, hydroxyurea, mitotane, aminoglutethimide; hormones, hormone analogs (estrogen, tamoxifen, goserelin, bicalutamide, nilutamide) and aromatase inhibitors (letrozole, anastrozole); anticoagulants (heparin, synthetic heparin salts and other inhibitors of thrombin); fibrinolytic agents (such as tissue plasminogen activator, streptokinase and urokinase), aspirin, dipyridamole, ticlopidine, clopidogrel, abciximab; antimigratory agents; antisecretory agents (breveldin); immunosuppressives (cyclosporine, tacrolimus (FK-506), sirolimus (rapamycin), azathioprine, mycophenolate mofetil); anti-angiogenic compounds (TNP-470, genistein) and growth factor inhibitors (vascular endothelial growth factor (VEGF) inhibitors, fibroblast growth factor (FGF) inhibitors); angiotensin receptor blocker; nitric oxide donors; anti-sense oligonucleotides; antibodies (trastuzumab); cell cycle inhibitors and differentiation inducers (tretinoin); mTOR inhibitors, topoisomerase inhibitors (doxorubicin (adriamycin), amsacrine, camptothecin, daunorubicin, dactinomycin, eniposide, epirubicin, etoposide, idarubicin and mitoxantrone, topotecan, irinotecan), corticosteroids (cortisone, dexamethasone, hydrocortisone, methylpednisolone, prednisone, and prenisolone); growth factor signal transduction kinase inhibitors; mitochondrial dysfunction inducers and caspase activators; and chromatin disruptors.

In certain embodiments, pharmaceutical compounds that may be used for combinatory anti-angiogenesis therapy include: (1) inhibitors of release of "angiogenic molecules," such as bFGF (basic fibroblast growth factor); (2) neutralizers of angiogenic molecules, such as an anti-βbFGF antibodies; and (3) inhibitors of endothelial cell response to angiogenic stimuli, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D_3 analogs, alpha-interferon, and the like. For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-

118 (1990), Moses et al., Science, 248:1408-1410 (1990), Ingber et al., Lab. Invest., 59:44-51 (1988), and U.S. Pat. Nos. 5,092,885, 5,112,946, 5,192,744, 5,202,352, and 6573256. In addition, there are a wide variety of compounds that can be used to inhibit angiogenesis, for example, peptides or agents that block the VEGF-mediated angiogenesis pathway, endostatin protein or derivatives, lysine binding fragments of angiostatin, melanin or melanin-promoting compounds, plasminogen fragments (e.g., Kringles 1-3 of plasminogen), tropoin subunits, antagonists of vitronectin $\alpha_v\beta_3$, peptides derived from Saposin B, antibiotics or analogs (e.g., tetracycline, or neomycin), dienogest-containing compositions, compounds comprising a MetAP-2 inhibitory core coupled to a peptide, the compound EM-138, chalcone and its analogs, and naaladase inhibitors. See, for example, U.S. Pat. Nos. 6,395,718, 6,462,075, 6,465,431, 6,475,784, 6,482,802, 6,482,810, 6,500,431, 6,500,924, 6,518,298, 6,521,439, 6,525,019, 6,538,103, 6,544,758, 6,544,947, 6,548,477, 6,559,126, and 6,569,845.

Depending on the nature of the combinatory therapy, administration of the polypeptide therapeutic agents of the invention may be continued while the other therapy is being administered and/or thereafter. Administration of the polypeptide therapeutic agents may be made in a single dose, or in multiple doses. In some instances, administration of the polypeptide therapeutic agents is commenced at least several days prior to the conventional therapy, while in other instances, administration is begun either immediately before or at the time of the administration of the conventional therapy.

VII. Methods of Administration and Pharmaceutical Compositions

In certain embodiments, the subject polypeptide therapeutic agents (e.g., soluble polypeptides or antibodies) of the present invention are formulated with a pharmaceutically acceptable carrier. Such therapeutic agents can be administered alone or as a component of a pharmaceutical formulation (composition). The compounds may be formulated for administration in any convenient way for use in human or veterinary medicine. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Formulations of the subject polypeptide therapeutic agents include those suitable for oral/ nasal, topical, parenteral, rectal, and/or intravaginal administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

In certain embodiments, methods of preparing these formulations or compositions include combining another type of anti-tumor or anti-angiogenesis therapeutic agent and a carrier and, optionally, one or more accessory ingredients. In general, the formulations can be prepared with a liquid carrier, or a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Formulations for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a subject polypeptide therapeutic agent as an active ingredient.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more polypeptide therapeutic agents of the present invention may be mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate;

(8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

In particular, methods of the invention can be administered topically, either to skin or to mucosal membranes such as those on the cervix and vagina. This offers the greatest opportunity for direct delivery to tumor with the lowest chance of inducing side effects. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl alcohol, dimethyl sulfoxide, and azone. Additional agents may further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples are salicylic acid and sulfur.

Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, and inhalants. The subject polypeptide therapeutic agents may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required. The ointments, pastes, creams and gels may contain, in addition to a subject polypeptide agent, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a subject polypeptide therapeutic agent, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Pharmaceutical compositions suitable for parenteral administration may comprise one or more polypeptide therapeutic agents in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic

agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

Injectable depot forms are made by forming microencapsule matrices of one or more polypeptide therapeutic agents in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

Formulations for intravaginal or rectally administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

In other embodiments, the polypeptide therapeutic agents of the instant invention can be expressed within cells from eukaryotic promoters. For example, a soluble polypeptide of EphB4 or Ephrin B2 can be expressed in eukaryotic cells from an appropriate vector. The vectors are preferably DNA plasmids or viral vectors. Viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the vectors stably introduced in and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression. Such vectors can be repeatedly administered as necessary. Delivery of vectors encoding the subject polypeptide therapeutic agent can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture et al., 1996, TIG., 12, 510).

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of

certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1. Soluble derivatives of the extracellular domains of human Ephrin B2 and EphB4 proteins

Soluble derivatives of the extracellular domains of human Ephrin B2 and EphB4 proteins represent either truncated full-length predicted extracellular domains of Ephrin B2 (B4ECv3, B2EC) or translational fusions of the domains with constant region of human immunoglobulins (IgG1 Fc fragment), such as B2EC-FC, B4ECv2-FC and B4ECv3-FC. Representative human Ephrin B2 constructs and human EphB4 constructs are shown Figures 14 and 15.

The cDNA fragments encoding these recombinant proteins were subcloned into mammalian expression vectors, expressed in transiently or stably transfected mammalian cell lines and purified to homogeneity as described in detail in Materials and Methods section (see below). Predicted amino acid sequences of the proteins are shown in Figures 1-5. High purity of the isolated proteins and their recognition by the corresponding anti-Ephrin B2 and anti-EphB4 monoclonal or polyclonal antibodies were confirmed. The recombinant proteins exhibit the expected high-affinity binding, binding competition and specificity properties with their corresponding binding partners as corroborated by the biochemical assays (see e.g., Figures 6-8).

Such soluble derivative proteins human Ephrin B2 and EphB4 exhibit potent biological activity in several cell-based assays and *in vivo* assays which measure angiogenesis or anti-cancer activities, and are therefore perspective drug candidates for anti-angiogenic and anti-cancer therapy. B4ECv3 as well as B2EC and B2EC-FC proteins blocked chemotaxis of human endothelial cells (as tested with umbilical cord and hepatic AECs or VECs), with a decrease in degradation of the extracellular matrix, Matrigel, and a decrease in migration in response to growth factor stimuli (Figures 9-11). B4ECv3 and B2EC-FC proteins have potent anti-angiogenic effect as demonstrated by their inhibition of endothelial cell tube formation (Figures 12-13).

Materials and Methods

1) Mammalian expression vectors for producing recombinant soluble derivatives of Ephrin B2 and Eph B4

Plasmids vectors for expressing recombinant soluble derivatives of Ephrin B2 and EphB4 were based on pEF6/V5-His-TOPO vector (Invitrogen), pIG (Novagen) or pRK5. pEF6/V5-His-TOPO contains human elongation factor 1á enhancer/promoter and blasticidin resistance marker. pIG vector is designed for high-level expression of protein fusions with Fc portion of human IgG1under CMV promoter control and pRK5 is a general purpose CMV promoter-containing mammalian expression vector. To generate plasmid construct pEF6-B4EC-NT, cDNA fragment of human EphB4 was amplified by PCR using oligo primers 5'-GGATCCGCC ATGGAGCTC CGGGTGCTGCT-3' and 5'-TGGATCCCT GCTCCCGC CAGCCCTCG CTCTCATCCA-3', and TOPO-cloned into pEF6/V5-His-TOPO vector. pEF6-hB4ECv3 was derived from pEF6-B4ECNT by digesting the plasmid DNA with EcoRV and BstBI, filling-in the ends with Klenow enzyme and religating the vector. Recombinant EphB4 derivative encoded by pEF6-B4EC-NT does not contain epitope- or purification tags, while the similar B4ECv3 protein encoded by pEF6-hB4ECv3 contains V5 epitope tag and 6xHis tag on its C-terminus to facilitate purification from conditioned media. Plasmid construct pEF6-hB2EC was created by PCR amplification of Ephrin B2 cDNA using oligo primers 5'- TGGATCCAC CATGGCTGT GAGAAGGGAC-3' plus 5'-ATTAATGGTGATGGT GAT GATGACTAC CCACTTCGG AACCGAGGATGTTGTTC-3' and TOPO-cloning into pEF6/V5-His-TOPO vector. Plasmid construct pIG-hB2EC-FC was created by PCR amplification of Ephrin B2 cDNA with oligo primers 5'-TAAAGCTTCCGCCATGG CTGTGAGAAGGGAC-3' and 5'-TAGGATCCACTTCGGA ACCGAGGATGTTGTT CCC-3' , followed by TOPO-cloning and sequencing the resulting PCR fragment with consecutive subcloning in pIG hIgG1 Fc fusion expression vector cut with Bam HI and Hind III. Similarly, pIG-hB2EC and pIG-hB4ECv3 were generated by PCR amplifying portions of EphB4 ECD cDNA using oligo primers 5'-ATAAGCTTCC GCCATGGAGC TCCGGGTGCTG-3' plus 5'-TTGGATCCTGCTCCCG CCAGCCCTCGC TCTCATC-3' with consecutive subcloning into pIG hIgG1 Fc fusion expression vector cut with Bam HI and Hind III. Predicted sequences of the proteins encoded by the vectors described above are shown in Figures 1-5.

2) Mammalian cell culture and transfections

HEK293T (human embryonic kidney line) cells were maintained in DMEM with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics. Cells were

maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. One day before transfections, 293T cells were seeded at a high density to reach 80% confluence at the time of transfection. Plasmid DNA and Lipofectamine reagent at 1:3 ratio were diluted in Opti-MEM I reduced serum medium (Invitrogen) for 5 min and mixed together to form DNA:Lipofectamine complex. For each 10 cm culture dish, 10 µg of plasmid DNA was used. After 20 min, above complex was added directly to cells in culture medium. After 16 hours of transfection, medium was aspirated, washed once with serum free DMEM and replaced with serum free DMEM. Secreted proteins were harvested after 48 hours by collecting conditional medium. Conditional medium was clarified by centrifugation at 10,000 g for 20 min, filtered through 0.2 µm filter and used for purification.

3) Generating stable cell lines

To create stable cell lines producing EphB4ECv3 and EphB4ECnt HEK293 or HEK293T cells were transfected with either pEF6-B4ECv3 or pEF6-B4EC-NT plasmid constructs as described above and selected using antibiotic Blasticidin. After 24 hours of transfection, cells were seeded at low density. Next day, cells were treated with 10 µg/ml of Blasticidin. After two weeks of drug selection, surviving cells were pooled and selected further for single cell clone expansion. After establishing stable cells, they were maintained at 4 µg/ml Blasticidin. Conditioned media were tested to confirm expression and secretion of the respective recombinant proteins. Specificity of expression was confirmed by Western blot with anti-B4 mono- or polyclonal ABs and B2EC-AP reagent binding and competition assays.

4) Protein purification

HEK293 cells were transiently transfected with a plasmid encoding secreted form of EphB4ectodomain (B4ECv3). Conditional media was harvested and supplemented with 10 mM imidazole, 0.3 M NaCl and centrifuged at 20,000g for 30 min to remove cell debris and insoluble particles. 80 ml of obtained supernatant were applied onto the pre-equilibrated column with 1 ml of Ni-NTA-agarose (Qiagen) at the flow rate of 10 ml/h. After washing the column with 10 ml of 50 mM Tris-HCl, 0.3 M NaCl and 10 mM imidazole, pH 8, remaining proteins were eluted with 3 ml of 0.25 M imidazole. Eluted proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl,

pH 8 overnight. Purity and identity of B4ECv3 was verified by PAGE/Coomassie G-250 and Western blot with anti-Eph.B4 antibody. Finally, the concentration of B4ECv3 was measured, and the protein was aliquoted and stored at -70 °C.

B4EC-FC protein and B2EC-FC protein were similarly purified.

5) Biochemical Assays

A. binding assay

10 µl of Ni-NTA-Agarose were incubated in microcentrifuge tubes with 50 µl of indicated amount of B4ECv3 diluted in binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin pH 8) After incubation for 30 min on shaking platform, Ni-NTA beads were washed twice with 1.4 ml of BB, followed by application of 50 µl of B2-AP in the final concentration of 50 nM. Binding was performed for 30 min on shaking platform, and then tubes were centrifuged and washed one time with 1.4 ml of BB. Amount of precipitated AP was measured colorimetrically after application of PNPP.

B. Iinhibition assay

Inhibition in solution. Different amounts of B4ECv3 diluted in 50 µl of BB were pre-incubated with 50 µl of 5 nM B2EC-AP reagent (protein fusion of Ephrin B2 ectodomain with placental alkaline phosphatase). After incubation for 1 h, unbound B2EC-AP was precipitated with 5,000 HEK293 cells expressing membrane-associated full-length EphB4 for 20 min. Binding reaction was stopped by dilution with 1.2 ml of BB, followed by centrifugation for 10 min. Supernatants were discarded and alkaline phosphatase activities associated with collected cells were measured by adding para-nitrophenyl phosphate (PNPP) substrate.

Cell based inhibition. B4ECv3 was serially diluted in 20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 8 and mixed with 5,000 HEK293 cells expressing membrane-associated full-length Ephrin B2. After incubation for 1 h, 50 µl of 5 nM B4EC-AP reagent (protein fusion of EphB4 ectodomain with placental alkaline phosphatase were added into each tube for 30 min to detect unoccupied Ephrin B2 binding sites. Binding reactions were stopped by dilution with 1.2

ml of BB and centrifugation. Colorimetric reaction of cell-precipitated AP was developed with PNPP substrate.

C. B4EC-FC binding assay

Protein A-agarose based assay. 10 µl of Protein A-agarose were incubated in Eppendorf tubes with 50 µl of indicated amount of B4EC-FC diluted in binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA pH 8). After incubation for 30 min on shaking platform, Protein AAagarose beads were washed twice with 1.4 ml of BB, followed by application of 50 µl of B2ECAP reagent at the final concentration of 50 nM. Binding was performed for 30 min on shaking platform, and then tubes were centrifuged and washed once with 1.4 ml of BB. Colorimetric reaction of precipitated AP was measured after application of PNPP (Fig. 6).

Nitrocellulose based assay. B4EC-FC was serially diluted in 20 mM Tris-HCl, 0.15 M NaCl, 50 µg/ml BSA, pH 8. 2 µl of each fraction were applied onto nitrocellulose strip and spots were dried out for 3 min. Nitrocellulose strip was blocked with 5% non-fat milk for 30 min, followed by incubation with 5 nM B2EC-AP reagent. After 45 min incubation for binding, nitrocellulose was washed twice with 20 mM Tris-HCl, 0.15 M NaCl, 50 µg/ml BSA, pH 8 and color was developed by application of alkaline phosphatase substrate Sigma Fast (Sigma).

D. B4EC-FC inhibition assay

Inhibition in solution. See above, for B4ECv3. The results were shown in Figure 7.

Cell based inhibition. See above, for B4ECv3.

E. B2EC-FC binding assay

Protein-A-agarose based assay. See above, for B4EC-FC. The results were shown in Figure 8.

Nitrocellulose based assay. See above, for B4EC-FC.

6) Cell-Based Assays

A. Growth Inhibition Assay

Human umbilical cord vein endothelial cells (HUVEC) (1.5x103) are plated in a 96-well plate in 100 µl of EBM-2 (Clonetic # CC3162). After 24 hours (day 0), the test recombinant protein (100 µl) is added to each well at 2X the desired concentration (5-7 concentration levels) in EBM-2 medium. On day 0, one plate is stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates are incubated for 72 h at 37 °C. After 72 h, plates are stained with 0.5% crystal violet in 20% methanol, rinsed with water and airdried. The stain is eluted with 1:1 solution of ethanol: 0.1 M sodium citrate (including day 0 plate), and absorbance is measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance is subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC50 (drug concentration causing 50% inhibition) is calculated from the plotted data.

B. Cord Formation Assay (Endothelial Cell Tube Formation Assay)

Matrigel (60 µl of 10 mg/ml; Collaborative Lab # 35423) is placed in each well of an ice-cold 96-well plate. The plate is allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit the matrigel to polymerize. In the mean time, HUVECs are prepared in EGM-2 (Clonetic # CC3162) at a concentration of $2X10^5$ cells/ml. The test compound is prepared at 2X the desired concentration (5 concentration levels) in the same medium. Cells (500 µl) and 2X drug (500 µl) is mixed and 200 µl of this suspension are placed in duplicate on the polymerized matrigel. After 24 h incubation, triplicate pictures are taken for each concentration using a Bioquant Image Analysis system. Drug effect (IC50) is assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

C. Cell Migration Assay

Migration is assessed using the 48-well Boyden chamber and 8 µm pore size collagen-coated (10 µg/ml rat tail collagen; Collaborative Laboratories) polycarbonate filters (Osmonics, Inc.). The bottom chamber wells receive 27-29 µl of DMEM medium alone (baseline) or medium containing chemo-attractant (bFGF, VEGF or Swiss 3T3 cell conditioned medium). The top chambers receive 45 µl of HUVEC cell suspension ($1X10^6$ cells/ml) prepared in DMEM+1% BSA with or without test compound. After 5 h incubation at 37 °C, the membrane is rinsed in

PBS, fixed and stained in Diff-Quick solutions. The filter is placed on a glass slide with the migrated cells facing down and cells on top are removed using a Kimwipe. The testing is performed in 4-6 replicates and five fields are counted from each well. Negative unstimulated control values are subtracted from stimulated control and drug treated values and data is plotted as mean migrated cell ± S.D. IC50 is calculated from the plotted data.

Example 2. Extracellular domain fragments of EphB4 receptor inhibit angiogenesis and tumor growth.

A. Globular domain of EphB4 is required for EphrinB2 binding and for the activity of EphB4-derived soluble proteins in endothelial tube formation assay.

To identify subdomain(s) of the ectopic part of EphB4 necessary and sufficient for the anti-angiogenic activity of the soluble recombinant derivatives of the receptor, four recombinant deletion variants of EphB4EC were produced and tested (Fig. 16). Extracellular part of EphB4, similarly to the other members of EphB and EphA receptor family, contains N-terminal ligand-binding globular domain followed by cysteine-rich domain and two fibronectin type III repeats (FNIII). In addition to the recombinant B4-GCF2 protein containing the complete ectopic part of EphB4, we constructed three deletion variants of EphB4EC containing globular domain and Cys-rich domain (B4-GC); globular, Cys-rich and the first FNIII domain (GCF1) as well as the ECD version with deleted globular domain (CF2). Our attempts to produce several versions of truncated EphB4EC protein containing the globular domain alone were not successful due to the lack of secretion of proteins expressed from all these constructs and absence of ligand binding by the intracellularly expressed recombinant proteins. In addition, a non-tagged version of B4-GCF2, called GCF2-F, containing complete extracellular domain of EphB4 with no additional fused amino acids was expressed, purified and used in some of the experiments described here.

All four C-terminally 6xHis tagged recombinant proteins were preparatively expressed in transiently transfected cultured mammalian cells and affinity purified to homogeneity from the conditioned growth media using chromatography on Ni^{2+}-chelate resin (Fig. 17). Apparently due to their glycosylation, the proteins migrate on SDS-PAAG somewhat higher than suggested by their predicted molecular weights of 34.7 kDa (GC), 41.5 (CF2), 45.6 kDa (GCF1) and 57.8 kDa (GCF2). Sequence of the extracellular domain of human EphB4 contains three predicted N-

glycosylation sites (NXS/T) which are located in the Cys-rich domain, within the first fibronectin type III repeat and between the first and the second fibronectin repeats.

To confirm ability of the purified recombinant proteins to bind Ephrin B2, they were tested in an *in vitro* binding assay. As expected, GC, GCF1 and GCF2, but not CF2 are binding the cognate ligand Ephrin B2 as confirmed by interaction between Ephrin B2 – alkaline phosphatase (Ephrin B2-AP) fusion protein with the B4 proteins immobilized on Ni^{2+}-resin or on nitrocellulose membrane (Fig. 17).

All four proteins were also tested for their ability to block ligand-dependent dimerization and activation of Eph B4 receptor kinase in PC3 cells. The PC3 human prostate cancer cell line is known to express elevated levels of human Eph B4. Stimulation of PC3 cells with Ephrin B2 IgG Fc fusion protein leads to a rapid induction of tyrosine phosphorylation of the receptor. However, preincubation of the ligand with GCF2, GCF1 or GC, but not CF2 proteins suppresses subsequent EphB4 autophosphorylation. Addition of the proteins alone to the PC3 cells or preincubation of the cells with the proteins followed by changing media and adding the ligand does not affect EphB4 phosphorylation status.

Further, we found that globular domain of EphB4 is required for the activity of EphB4-derived soluble proteins in endothelial tube formation assay.

B. Effects of soluble EphB4 on HUV/AEC *in vitro*.

Initial experiments were performed to determine whether soluble EphB4 affected the three main stages in the angiogenesis pathway. These were carried out by establishing the effects of soluble EphB4 on migration / invasion, proliferation and tubule formation by HUV/AEC *in vitro*. Exposure to soluble EphB4 significantly inhibited both bFGF and VEGF-induced migration in the Boyden chamber assay in a dose-dependent manner, achieving significance at nM (Fig. 18). Tubule formation by HUV/AECS on wells coated with Matrigel was significantly inhibited by soluble EphB4 in a dose-dependent manner in both the absence and presence of bFGF and VEGF (Fig. 19). We also assessed *in vitro,* whether nM of soluble EphB4 was cytotoxic for HUVECS. Soluble EphB4 was found to have no detectable cytotoxic effect at these doses, as assessed by MTS assay (Fig. 20).

C. Soluble EphB4 receptor Inhibits Vascularization of Matrigel Plugs, in vivo

To demonstrate that soluble EphB4 can directly inhibit angiogenesis *in vivo*, we performed a murine matrigel plug experiment. Matrigel supplemented with bFGF and VEGF with and without soluble EphB4 was injected s.c. into Balb/C nu/nu mice, forming semi-solid plugs, for six days. Plugs without growth factors had virtually no vascularization or vessel structures after 6 days (Fig. 21). In contrast, plugs supplemented with bFGF and VEGF had extensive vascularization and vessels throughout the plug. Plugs taken from mice treated with μg of soluble EphB4 had markedly reduced vascularization of plugs, comparable to plugs without growth factor (Fig. 21). Furthermore, histological examination of plugs showed decreased vessel staining (Fig. 21). Treatment at 0 μg/dose significantly inhibited the amount of infiltration in Matrigel plugs compared to control (Fig. 21).

We examined EphB4 receptor phosphorylation in HUVECs by performing Western blot analyses with lysates from soluble EphB4-treated cells and antibodies against phosphor-tyrosine. We found that soluble EphB4 treatment of serum-starved HUVECs stimulated a rapid and transient decrease in the level of phosphorylated EphB4, in the presence of EphrinB2Fc, EphB4 ligand dimer. Ephrin B2Fc without the soluble EphB4 protein induced phosphorylation of EphB4 receptor (Fig. 22).

D. Effects of soluble EphB4 on tumor growth, *in vitro.*

We found that soluble EphB4 inhibits the growth of SCC15 tumors grown in Balb/C Nu/Nu mice (Fig. 23).

E. Soluble EphB4 inhibited corneal neovascularization

To further investigate the antiangiogenic activity of soluble EphB4 in vivo, we studied the inhibitory effect of administration of soluble EphB4 on neovascularization in the mouse cornea induced by bFGF. Hydron Pellets implanted into corneal micropocket could induce angiogenesis, in the presence of growth factors, in a typically avascular area. The angiogenesis response in mice cornea was moderate, the appearance of vascular buds was delayed and the new capillaries were sparse and grew slowly. Compared with the control group, on day 7 of implantation, the neovascularization induced by bFGF in mice cornea was markedly inhibited in soluble EphB4-treated group (Fig. 24).

F. Effects of soluble EphB4 on tumor growth, *in vivo.*

The same model was used to determine the effects of soluble EphB4 *in vivo*. SCC15 tumors implanted subcutaneously, pre-incubated with matrigel and with or w/o growth factors, as well as implanted sc alone, and mice treated sc or ip daily with 1-5ug of soluble EphB4 were carried out.

Tumors in the control group continued to grow steadily over the treatment period, reaching a final tumor volume of mm3. However, animals injected with soluble EphB4 exhibited a significantly ($p<0.0/$) reduced growth rate, reaching a final tumor volume of only mm3 (Fig. 25). Similar results were obtained in two further cohorts of such tumor-bearing mice. Soluble EphB4 administration appeared to be well tolerated *in vivo*, with no significant effect on body weight or the general well-being of the animals (as determined by the absence of lethargy, intermittent hunching, tremors or disturbed breathing patterns).

G. Effects of soluble EphB4 on tumor histology.

Histological analysis revealed the presence of a central area of necrosis in all SCC15 tumors, which was usually surrounded by a viable rim of tumor cells um in width. The central necrotic areas were frequently large and confluent and showed loss of cellular detail. Necrosis, assessed as a percentage of tumor section area, was significantly ($p<0.02$) more extensive in the soluble EphB4-treated group (% necrosis in treated vs. control). To determine whether the reduced volume of soluble EphB4 treated tumors was due to an effect of this protein on the tumor vascular supply, endothelial cells in blood vessels were identified in tumor sections using immunostaining with an anti-platelet cell adhesion molecule (PECAM-1; CD31) antibody (Fig. 26) and the density of microvessels was assessed. Microvessel density was similar in the outer viable rim of tumor cells (the uniform layer of cells adjacent to the tumor periphery with well defined nuclei) in control and soluble EphB4-treated tumors. Microvessel density was significantly in the inner, less viable region of tumor cells abutting the necrotic central areas in soluble EphB4-treated than control tumors. Fibrin deposition, as identified by Masson's Trichrome staining, was increased in and around blood vessels in the inner viable rim and the central necrotic core of soluble EphB4 treated than control tumors. In the outer viable rim of soluble EphB4 treated tumors, although the vessel lumen remained patent and contained red blood cells, fibrin deposition was evident around many vessels. Soluble EphB4 was found to

have no such effects on the endothelium in the normal tissues examined (lungs, liver and kidneys).

H. Materials and Methods

1) Expression constructs

To construct expression vectors for producing soluble, 6xHis-tagged EphB4-ECD variants, cloned full-length human EphB4 cDNA was amplified by PCR using the following oligo primers: TACTAGTCCGCCATGGAGCTCCGGGTGCTGCT (common EphB4 N-terminal primer) and GCGGCCGCTTAATGGTGATGGTGA TGATGAGCCGAAGGA GGGGTGGTGCA (B4-GC), AGCGGCCGCTTAATGGTGATGGTGAT GATGGACATTGA CAGGCTCAAATGGGA (B4-GCF1) or TGCGGCCGCTTAATGGTGATGGTGATGAT GCTGCTCCCGCCAGCCCTCGCTCTCAT (B4-GCF2). The resulting PCR fragments were TA-cloned into mammalian expression vector pEF6/V5-His-TOPO (Invitrogen) under EF-1α promoter control. The expressed recombinant proteins encode the following fragments of the mature extracellular part of human EphB4: amino acid positions 1-522 (GCF2), 1-412 (GCF1) and 1-312 (GC). To generate the B4-CF2 deletion (δ amino acids 13-183) PCR fragment for pEF6 cloning, EphB4 cDNA was amplified by two-step overlap PCR using oligo primers TACTAGTCCGCCATGGAGCTCCGGGTGCTGCT, CAGCTGAGTTTCCAATTTTGTGTTC, GAACACAAAATTGGAAACTCAGCTGACTGTGAACCTGAC and GCGGCCGCCCTG CTCCCGCCAGCCCTCGCT.

Vector for producing secreted human EphrinB2-alkaline phosphatase (B2-AP) reagent was constructed by PCR amplification of human Ephrin B2 cDNA using primers TAAAGCTTCCGCCATGGCTGTGAGAAGGGAC and TAGGATCCTTCGGAACCG AGGATGTTGTTCCC and cloning the resulting fragment, digested with Hind III and Bam HI, into Hind III-Bgl II digested pAPTag2 vector (GenHunter, Inc.). In each case, inserts in expression vectors were verified by complete sequencing.

2) Antibodies and other reagents

Anti-Eph B4 monoclonal antibodies mAB79 and mAB23 were raised in mice against the GCF2 protein containing amino acids 1-522 of mature human EphB4 and purified from

hybridoma supernatants by Protein A chromatography. The anti-phosphotyrosine antibody 4G10 was from UBI (Lake Placid, NY). Protein G-HRP conjugate was purchased from Bio-Rad.

3) Expression and purification of EphB4-derived recombinant proteins

To produce the EphB4-ECD soluble proteins, cultured human embryonic kidney cells HEK293T were transfected with the corresponding plasmid constructs using standard calcium phosphate or Lipofectamin 2000 reagent (Invitrogen) protocols. Twelve to sixteen hours post-transfection, the growth medium (DMEM+10% fetal bovine serum) was aspirated, cells washed once with serum free DMEM and replaced with serum free DMEM. Conditioned media containing the secreted proteins were harvested 72-96 hours later, clarified by centrifugation and used for purification of His-tagged proteins using Ni-NTA Agarose (Qiagen). The purity and quantity of the recombinant proteins was tested by SDS-PAAG electrophoresis with Coomassie Blue or silver staining, Western blotting and UV spectroscopy. Purified proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 8 and stored at -70 °C.

To test ligand binding properties of the proteins, 10 µl of Ni-NTA-Agarose (Qiagen) were incubated in microcentrifuge tubes with 10-500 ng sample of a B4-ECD protein diluted in 0.5 ml of binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin, pH 8). After incubation for 30 min on shaking platform, Ni-NTA beads were washed twice with 1.4 ml of BB, followed by addition of B2-AP fusion protein at concentration of 50 nM. Binding was performed for 30 min on a shaking platform. Tubes were centrifuged and washed once with 1.4 ml of BB. Amount of precipitated AP was measured colorimetrically at 420 nm after application of p-nitrophenyl phosphate (PNPP) and incubation for 5-30 min.

4) Immunoprecipitation

All lysates were processed at 4 °C. Cells were lysed in 1 ml of buffer containing 20 mM Hepes at pH 7.4, 100 mM sodium chloride, 50 mM sodium fluoride, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 1%(v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM phenyl methylsulphonyl fluoride (added freshly) and 100U Trasylol. Lysates were scraped into Eppendorf tubes and 50 µl of boiled, formalin-fixed *Staphylococcus aureus* was added (Calbiochem, San Diego). After 30 min of mixing, the lysates were centrifuged for 5 min at 25,000*g* in a minifuge and the supernatants transferred to new tubes containing the appropriate

antibody. Lysates were mixed with antibodies for 1 h, after which time 50 µl of protein A–Sepharose beads were added and the contents of the tubes mixed for 1 h to collect the immunoprecipitates. Protein A beads were collected by centrifugation at 25,000*g* for 30 s. The supernatants were discarded and the beads washed three times with 1 ml lysis buffer minus deoxycholate.

5) Cell-based EphB4 tyrosine kinase assay

The human prostate carcinoma cell line PC3 cells were maintained in RPMI medium with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics mix. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Typically, cells were grown in 60 mm dishes until confluency and were either treated with mouse Ephrin B2-Fc fusion at 1 µg/ml in RPMI for 10 min to activate EphB4 receptor or plain medium as a control. To study the effect of different derivatives of soluble EphB4 ECD proteins on EphB4 receptor activation, three sets of cells were used. In the first set, cells were treated with various proteins (5 proteins; GC, GCF1, GCF2, GCF2-F, CF2) at 5 µg/ml for 20 min. In the second set of cells, prior to application, proteins were premixed with ephrinB2-Fc at 1:5 (EphB4 protein: B2-Fc) molar ratio, incubated for 20 min and applied on cells for 10 min. In the third set of cells, cells were first treated with the proteins for 20 min at 5 µg/ml, media was replaced with fresh media containing 1 µg/ml of EphrinB2-Fc and incubated for another 10 min.

After the stimulation, cells were immediately harvested with protein extraction buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X100, 1 mM EDTA, 1 mM PMSF, 1 mM Sodium vanadate. Protein extracts were clarified by centrifugation at 14,000 rpm for 20 min at 4 °C. Clarified protein samples were incubated overnight with protein A/G coupled agarose beads pre-coated with anti-EphB4 monoclonal antibodies. The IP complexes were washed twice with the same extraction buffer containing 0.1% Triton X100. The immunoprecipitated proteins were solubilized in 1X SDS-PAGE sample loading buffer and separated on 10% SDS-PAGE. For EphB4 receptor activation studies, electroblotted membrane was probed with anti-pTyr specific antibody 4G10 at 1:1000 dilution followed by Protein G-HRP conjugate at 1:5000 dilutions.

6) Cell Culture

Normal HUVECs were obtained from Cambrex (BioWhittaker) and maintained in EBM2 medium supplemented with 0.1 mg/ml endothelial growth supplement (crude extract from bovine brain), penicillin (50 U/ml), streptomycin (50 U/ml), 2 mmol/l glutamine and 0.1 mg/ml sodium heparin. Aliquots of cells were preserved frozen between passages 1 and 3. For all experiments, HUVECs were used at passages 4 or below and collected from a confluent dish.

7) Endothelial Cell Tube Formation Assay

Matrigel (60 µl of 10mg/ml; Collaborative Lab, Cat. No. 35423) was placed in each well of an ice-cold 96-well plate. The plate was allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit Matrigel to polymerize. In the mean time, human umbilical vein endothelial cells were prepared in EGM-2 (Clonetic, Cat. No. CC3162) at a concentration of $2x10^5$ cells/ml. The test protein was prepared at 2x the desired concentration (5 concentration levels) in the same medium. Cells (500 µl) and 2x protein (500 µl) were mixed and 200 µl of this suspension were placed in duplicate on the polymerized Matrigel. After 24 h incubation, triplicate pictures were taken for each concentration using a Bioquant Image Analysis system. Protein addition effect (IC_{50}) was assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

8) Cell Migration Assay

Chemotaxis of HUVECs to VEGF was assessed using a modified Boyden chamber, transwell membrane filter inserts in 24 well plates, 6.5 mm diam, 8 µm pore size, 10 µm thick matrigel coated, polycarbonate membranes (BD Biosciences). The cell suspensions of HUVECs ($2x\ 10^5$ cells/ml) in 200 µl of EBM were seeded in the upper chamber and the soluble EphB4 protein were added simultaneously with stimulant (VEGF or bFGF) to the lower compartment of the chamber and their migration across a polycarbonate filter in response to10- 20 ng/ml of VEGF with or without 100 nM-1 µM test compound was investigated. After incubation for 4-24 h at 37 °C, the upper surface of the filter was scraped with swab and filters were fixed and stained with Diff Quick. Ten random fields at 200x mag were counted and the results expressed as mean # per field. Negative unstimulated control values were subtracted from stimulated control and protein treated sample values and the data was plotted as mean migrated cell ± S.D. IC_{50} was calculated from the plotted data.

9) Growth Inhibition Assay

HUVEC ($1.5x10^3$ cells) were plated in a 96-well plate in 100 µl of EBM-2 (Clonetic, Cat. No. CC3162). After 24 hours (day 0), the test recombinant protein (100 µl) is added to each well at 2x the desired concentration (5-7 concentration levels) in EBM-2 medium. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates were incubated for 72 h at 37 °C. After 72 h, plates were stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain was eluted with 1:1 solution of ethanol: 0.1M sodium citrate (including day 0 plate), and absorbance measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance was subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC_{50} value was calculated from the plotted data.

10) Murine Matrigel Plug Angiogenesis Assay

In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into a Matrigel plug containing the test sample. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. Matrigel (8.13 mg/ml, 0.5 ml) in liquid form at 4 °C was mixed with Endothelial Cell Growth Supplement (ECGS), test proteins plus ECGS or Matrigel plus vehicle alone (PBS containing 0.25% BSA). Matrigel (0.5ml) was injected into the abdominal subcutaneous tissue of female nu/nu mice (6 wks old) along the peritoneal mid line. There were 3 mice in each group. The animals were cared for in accordance with institutional and NIH guidelines. At day 6, mice were sacrificed and plugs were recovered and processed for histology. Typically the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin in PBS and embedded in paraffin. Sections of 3 µm were cut and stained with H&E or Masson's trichrome stain and examined under light microscope

11) Mouse Corneal Micropocket assay

Mouse corneal micropocket assay was performed according to that detailed by Kenyon et al., 1996. Briefly, hydron pellets (polyhydroxyethylmethacrylate [polyHEMA], Interferon Sciences, New Brunswick, NJ, U.S.A.) containing either 90 ng of bFGF (R&D) or 180 ng of VEGF (R&D Systems, Minneapolis, MN, U.S.A.) and 40 µg of sucrose aluminium sulfate (Sigma) were prepared. Using an operating microscope, a stromal linear keratotomy was made with a surgical blade (Bard-Parker no. 15) parallel to the insertion of the lateral rectus muscle in

an anesthetized animal. An intrastromal micropocket was dissected using a modified von Graefe knife (2¨30 mm). A single pellet was implanted and advanced toward the temporal corneal limbus (within 0±7±1±0 mm for bFGF pellets and 0±5 mm for VEGF pellets). The difference in pellet location for each growth factor was determined to be necessary given the relatively weaker angiogenic stimulation of VEGF in this model. Antibiotic ointment (erythromycin.) was then applied to the operated eye to prevent infection and to decrease surface irregularities. The subsequent vascular response was measured extending from the limbal vasculature toward the pellet and the contiguous circumferential zone of neovascularization Data and clinical photos presented here were obtained on day 6 after pellet implantation, which was found to be the day of maximal angiogenic response.

12) In vitro invasion assay

"Matrigel" matrix-coated 9-mm cell culture inserts (pore size, 8 μm; Becton Dickinson, Franklin Lakes, NJ) were set in a 24-well plate. The HUVEC cells were seeded at a density of $5x10^3$ cells per well into the upper layer of the culture insert and cultured with serum-free EBM in the presence of EphB4 ECD for 24 h. The control group was cultured in the same media without EphB4. Then 0.5 ml of the human SCC15 cell line, conditioned medium was filled into the lower layer of the culture insert as a chemo-attractant. The cells were incubated for 24 h, then the remaining cells in the upper layer were swabbed with cotton and penetrating cells in the lower layer were fixed with 5% glutaraldehyde and stained with Diff Quick. The total number of cells passing through the Matrigel matrix and each 8 μm pore of the culture insert wascounted using optical microscopy and designated as an invasion index (cell number/area).

13) SCC15 tumor growth in mice

Subcutaneously inject logarithmically growing SCC15, head and neck squamous cell carcinoma cell line, at $5X10^6$ cell density; with or without EphB4 ECD in the presence or absence of human bFGF, into athymic Balb/c nude mice, along with Matrigel (BD Bioscience) synthetic basement membrane (1:1 v/v), and examine tumors within 2 weeks. Tumor volumes in the EphB4 ECD group, in the presence and absence of growth factor after implantation were three-fold smaller than those in the vehicle groups. There was no difference in body weight between the groups. Immunohistochemical examination of cross-sections of resected tumors and TUNEL-positive apoptosis or necrosis, CD34 immunostaining, and BrdU proliferation rate will

be performed, after deparaffinized, rehydrated, and quenched for endogenous peroxidase activity, and after 10 min permeabilization with proteinase K. Quantitative assessment of vascular densities will also be performed. Local intratumoral delivery or IV delivery of EphB4 ECD will also be performed twice a week.

30 athymic nude mice, BALB/c (nu/nu), were each injected with 1 x 10^6 B16 melanoma cells with 0.1 ml PBS mixed with 0.1 ml matrigel or 1.5 x 10^6 SCC15 cells resuspended in 200 µl of DMEM serum-free medium and injected subcutaneously on day 0 on the right shoulder region of mice. Proteins were injected intravenously or subcutaneously, around the tumor beginning on day 1 at a loading dose of 4 µg/mg, with weekly injections of 2ug/mg. (10 µg/g, 50 µg/kg/day), and at 2 weeks post-inoculation. Mice are sacrificed on Day 14. Control mice received PBS 50 µl each day.

14) Tumor formation in nude mice

All animals were treated under protocols approved by the institutional animal care committees. Cancer cells ($5x10^6$) were subcutaneously inoculated into the dorsal skin of nude mice. When the tumor had grown to a size of about 100 mm^3 (usually it took ~12 days), sEphB4 was either intraperitoneally or subcutaneously injected once/day, and tumorigenesis was monitored for 2 weeks. Tumor volume was calculated according to the formula a^2xb, where a and b are the smallest and largest diameters, respectively. A Student's t test was used to compare tumor volumes, with $P<.05$ being considered significant.

15) Quantification of microvessel density

Tumors were fixed in 4% formaldehyde, embedded in paraffin, sectioned by 5 µm, and stained with hematoxylin~eosin. Vessel density was semi-quantitated using a computer-based image analyzer (five fields per section from three mice in each group).

Example 3. EphB4 Is Upregulated and Imparts Growth Advantage in Prostate Cancer

A. Expression of EphB4 in prostate cancer cell lines

We first examined the expression of EphB4 protein in a variety of prostate cancer cell lines by Western blot. We found that prostate cancer cell lines show marked variation in the abundance of the 120 kD EphB4. The levels were relatively high in PC3 and even higher in

PC3M, a metastatic clone of PC3, while normal prostate gland derived cell lines (MLC) showed low or no expression of EphB4 (Fig. 27A). We next checked the activation status of EphB4 in PC3 cells by phosphorylation study. We found that even under normal culture conditions, EphB4 is phosphorylated though it can be further induced by its ligand, ephrin B2 (Fig. 27B).

B. Expression of EphB4 in clinical prostate cancer samples

To determine whether EphB4 is expressed in clinical prostate samples, tumor tissues and adjacent normal tissue from prostate cancer surgical specimens were examined. The histological distribution of EphB4 in the prostate specimens was determined by immunohistochemistry. Clearly, EphB4 expression is confined to the neoplastic epithelium (Fig. 28, top left), and is absent in stromal and normal prostate epithelium (Fig. 28, top right). In prostate tissue array, 24 of the 32 prostate cancers examined were positive. We found EphB4 mRNA is expressed both in the normal and tumor tissues of clinical samples by quantitative RT-PCR. However, tumor EphB4 mRNA levels were at least 3 times higher than in the normal in this case (Fig. 28, lower right).

C. p53 and PTEN inhibited the expression of EphB4 in PC3 cells

PC3 cells are known to lack PTEN expression (Davis, et al., 1994, Science. 266:816-819) and wild-type p53 function (Gale, et al., 1997, Cell Tissue Res. 290:227-241). We investigated whether the relatively high expression of EphB4 is related to p53 and/or PTEN by re-introducing wild-type p53 and/or PTEN into PC3 cells. To compensate for the transfection efficiency and the dilution effect, transfected cells were sorted for the cotransfected truncated CD4 marker. We found that the expression of EphB4 in PC3 cells was reduced by the re-introduction of either wild-type p53 or PTEN. The co-transfection of p53 and PTEN did not further inhibit the expression of EphB4 (Fig. 29A).

D. Retinoid X receptor (RXR α) regulates the expression of EphB4

We previously found that RXRα was down-regulated in prostate cancer cell lines (Zhong, et al., 2003, Cancer Biol Ther. 2:179-184) and here we found EphB4 expression has the reverse expression pattern when we looked at "normal" prostate (MLC), prostate cancer (PC3), and metastatic prostate cancer (PC3M) (Fig. 27A), we considered whether RXRα regulates the expression of EphB4. To confirm the relationship, the expression of EphB4 was compared

between CWR22R and CWR22R-RXRα, which constitutively expresses RXRα. We found a modest decrease in EphB4 expression in the RXRα overexpressing cell line, while FGF8 has no effect on EphB4 expression. Consistent with initial results, EphB4 was not found in "normal" benign prostate hypertrophic cell line BPH-1 (Fig. 29B).

E. Growth factor signaling pathway of EGFR and IGF-1R regulates EphB4 expression

EGFR and IGF-1R have both been shown to have autocrine and paracrine action on PC3 cell growth. Because we found that EphB4 expression is higher in the more aggressive cell lines, we postulated that EphB4 expression might correlate with these pro-survival growth factors. We tested the relationship by independently blocking EGFR and IGF-1R signaling. EphB4 was down-regulated after blocking the EGFR signaling using EGFR kinase inhibitor AG 1478 (Fig. 30A) or upon blockade of the IGF-1R signaling pathway using IGF-1R neutralizing antibody (Fig. 30B).

F. EphB4 siRNA and antisense ODNs inhibit PC3 cell viability

To define the significance of this EphB4 overexpression in our prostate cancer model, we concentrated our study on PC3 cells, which have a relatively high expression of EphB4. The two approaches to decreasing EphB4 expression were siRNA and AS-ODNs. A number of different phosphorothioate-modified AS-ODNs complementary to different segments of the EphB4 coding region were tested for specificity and efficacy of EphB4 inhibition. Using 293 cells transiently transfected with full-length EphB4 expression vector AS-10 was found to be the most effective (Fig. 31B). A Similar approach was applied to the selection of specific siRNA. EphB4 siRNA 472 effectively knocks down EphB4 protein expression (Fig. 31A). Both siRNA 472 and antisense AS-10 ODN reduced the viability of PC3 cells in a dose dependent manner (Fig. 31C, D). Unrelated siRNA or sense oligonucleotide had no effect on viability.

G. EphB4 siRNA and antisense ODNs inhibit the mobility of PC3 Cells

PC3 cells can grow aggressively locally and can form lymph node metastases when injected orthotopically into mice. In an effort to study the role of EphB4 on migration of PC3 cells *in vitro*, we performed a wound-healing assay. When a wound was introduced into a monolayer of PC3 cells, over the course of the next 20 hours cells progressively migrated into the cleared area. However, when cells were transfected with siRNA 472 and the wound was

introduced, this migration was significantly inhibited (Fig. 31E). Pretreatment of PC3 cells with 10 μM EphB4 AS-10 for 12 hours generated the same effect (Fig. 31F). In addition, knock-down of EphB4 expression in PC3 cells with siRNA 472 severely reduced the ability of these cells to invade Matrigel as assessed by a double-chamber invasion assay (Fig. 31G), compared to the control siRNA.

H. EphB4 siRNA induces cell cycle arrest and apoptosis in PC3 cells

Since knock-down of EphB4 resulted in decreased cell viability (Fig. 31C) we sought to determine whether this was due to effects on the cell cycle. In comparison to control siRNA transfected cells, siRNA 472 resulted in an accumulation of cells in the sub G0 and S phase fractions compared to cells treated with control siRNA. The sub G0 fraction increased from 1 % to 7.9%, and the S phase fraction from 14.9 % to 20.8 % in siRNA 472 treated cells compared to control siRNA treated cells (Fig. 32A). Cell cycle arrest at sub G0 and G2 is indicative of apoptosis. Apoptosis as a result of EphB4 knock-down was confirmed by ELISA assay. A dose-dependent increase in apoptosis was observed when PC3 cells were transfected with siRNA 472, but not with control siRNA (Fig. 32B). At 100 nM there was 15 times more apoptosis in siRNA 472 transfected than control siRNA transfected PC3 cells.

I. Materials and Methods

1) Reagents

Neutralizing IGF-1R antibody was from R&D Systems (Minneapolis MN). Anti-IGF-1R(β), -EGFR, -EphB4(C-16) were from Santa Cruz Biotech (Santa Cruz, CA). β-actin monoclonal antibody was purchased from Sigma Chemical Co. (St Louis, MO). Media and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). AG 1478(4-(3'-Chloroanilino)-6,7-dimethoxy-quinazoline) was from Calbiochem (San Diego, CA).

2) Antisense oligodeoxynucleotides and EphB4 siRNAs

EphB4 specific antisense phosphorothioate-modified oligodeoxynucleotide (ODN) and sense ODN were synthesized and purified by Qiagen (Alameda CA). The sequences are: Sense, 5'-TCC-TGC-AAG-GAG-ACC-TTC-AC-3'; AS1: 5'-GTG-CAG-GGA-TAG-CAG-GGC-CAT-3'; AS10: 5'-ATG-GAG-GCC-TCG-CTC-AGA-AA-3'. siRNAs were synthesized at the USC/Norris Comprehensive Cancer Center Microchemical Core laboratory. Sequences of EphB4

siRNAs are siRNA 472 5'-GGU-GAA-UGU-CAA-GAC-GCU-GUU-3' and siRNA 2303 5'-cuc-uuc-cga-ucc-cac-cua-cuu-3'. Negative control siRNA to scrambled GAPDH was from Ambion (Austin, TX)

3) Cell lines and culture

The prostate cancer cell lines, PC3, PC3M, DU145, ALVA31, LAPC-4, LNCaP, CWR22R and adult human normal prostate epithelial cell line MLC SV40, and BPH-1 were obtained and cultured as described previously (7). Stable cell line CWR22R-RXR, LNCaP-FGF8 were established and cultured as described before (7, 33).

4) Generation of EphB4 monoclonal antibody

The extracellular domain (ECD) of EphB4 was cloned into pGEX-4T-1 to generate GST-fused ECD (GST-ECD). EphB4ECD expressed as a GST fusion protein in BL21 *E. coli* was purified by affinity chromatography and the GST domain was cleaved by thrombin. Monoclonal antibody was generated and the sensitivity and specificity of the antibody was reconfirmed by Western blot with whole cell lysate of 293 cells stably transfected with EphB4.

5) One-Step RT-PCR and Quantitative RT-PCR

Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc. Friendswood TX) from prostate cancer specimens and adjacent normal specimens. For quantitative RT-PCR first strand cDNA was synthesized from 5 μg of total RNA using SuperScript III (Invitrogen, Carlsbad CA). Quantitative RT-PCR was performed on the Stratagene MX3000P system (Stratagene, La Jolla CA) using SYBR Green I Brilliant Mastermix (Stragene) according to the manufacture's instructions. Optimized reactions for EphB4 and β-actin (used as the normalizer gene) were 150 nM each of the forward primer (β-actin, 5'-GGA-CCT-GAC-TGA-CTA-CCT-A-3'; EphB4, 5'-AAG-GAG-ACC-TTC-ACC-GTC-TT-3') and reverse primer (β-actin 5'-TTG-AAG-GTA-GTT-TCG-TGG-AT-3'; EphB4, 5'-TCG-AGT-CAG-GTT-CAC-AGT-CA-3') with DNA denaturation/activation of polymerase at 95 °C for 10 min followed by 40 cycles of 95 °C for 30s, 60 °C for 1min, 72 °C for 1min. The specificity of the gene-specific amplification was confirmed by the presence of a single dissociation peak. All reactions were performed in triplicate with RT and no template negative controls.

6) Immunohistochemistry

OCT-embedded tissues were sectioned at 5 μm and fixed in phosphate-buffered 4% paraformaldehyde. Sections were washed for 3 x 5 min in PBS and endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in PBS for 10 min at room temperature. Sections were incubated with Eph4 (C-16) antibody (1:50) for 1 h at room temperature followed by three washes in PBS and incubation with donkey anti-goat secondary antibody (Santa Cruz Biotech.) for 1 h at room temperature. After three washes in PBS, peroxidase activity was localized by incubation in DAB substrate solution (Vector Laboratories, Inc. Burlingame CA) for 10 min at room temperature. Sections were counterstained with Hematoxylin for 20 s, dehydrated and mounted. Negative control for staining was substitution of normal goat serum for primary antibody. Immunohistochemical staining on prostate array (BioMeda, Foster City, CA) was done using goat ABC Staining System (Santa Cruz Biotech.) according to the manufacturer's instructions.

7) Western blot

Whole cell lysates were prepared using Cell Lysis Buffer (GeneHunter, Basgvukke TN) supplemented with protease inhibitor cocktail (Pierce, Rockford IL), unless otherwise noted. Total protein was determined using the DC reagent system (Bio-Rad, Hercules CA). Typically, 20 μg whole cell lysate was run on 4-20% Tris-Glycine gradient gel. The samples were electro-transferred to PVDF membrane and the non-specific binding was blocked in TBST buffer (0.5 mM Tris-HCl, 45 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% non-fat milk. Membranes were first probed with primary antibody overnight, stripped with Restore™ Western Blot stripping buffer (Pierce, Rockford IL) and reprobed with β-actin to confirm equivalent loading and transfer of protein. Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

8) Phosphorylation analysis

Cells growing in 60 mm dishes were either serum starved (1% FBS supplemented RPMI 1640, 24 hours) or cultured in normal conditions (10% FBS) and then treated with or without 1 μg/ml mouse ephrin B2/F_c for 10 min to activate EphB4 receptor Cleared cell lysates were incubated with EphB4 monoclonal antibody overnight at 4 °C. Antigen-antibody complex was immunoprecipitated by the addition of 100 μl of Protein G-Sepharose in 20 mM sodium phosphate, pH 7.0 with incubation overnight at 4 °C. Immunoprecipitates were analyzed by

Western blot with pTyr specific antibody (Upstate, clone 4G10) at 1:1000 dilution followed by incubation with protein G-HRP (Bio-Rad) at 1:5000 dilution. To monitor immunoprecipitation efficiency, a duplicate membrane was probed with EphB4 specific monoclonal antibody.

9) Transient transfection and sorting of transfected cells

PC3 cells were cotransfected with pMACS 4.1 coding for CD4 and wild type p53 (pC53-SN3) or PTEN vector or both using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The molar ratio of CD4 to p53 or PTEN or vector was 1:3 and total plasmid was 24 μg for a 10 cm^2 dish of 90% confluent cells using 60 μl of Lipofectamine 2000. 24 hours after transfection, a single cell suspension was made and sorted using truncated CD4 as a surface marker according to the manufacturer's protocol (Miltenyi Biotec, Germany). Sorted cells were lysed in 1 x SDS sampling buffer and analyzed by Western blot.

10) Study of IGF and EGF signaling pathway on the expression of EphB4

PC3 cells were seeded into 6-well plates and cultured until 80% confluent and treated with 2 μg/ml neutralizing IGF-1R monoclonal antibody, MAB391 (Hailey, et al., 2002, Mol Cancer Ther. 1:1349-1353), or with 1 nM AG 1478, a strong EGFR inhibitor (Liu, et al., 1999, J Cell Sci. 112 (Pt 14):2409-2417) for 24 h. Crude cell lysates were analyzed by Western blot. Band density was quantified with the Bio-Rad QuantityOne System software.

11) Cell viability assay

PC3 cells were seeded on 48-well plates at a density of approximately 1×10^4 cells/well in a total volume of 200 ml. Media was changed after the cells were attached and the cells were treated with various concentrations (1-10 μM) of EphB4 antisense ODN or sense ODN as control. After three days media was changed and fresh ODNs added. Following a further 48 h incubation, cell viability was assessed by MTT as described previously (36). EphB4 siRNAs (10-100 nM) were introduced into 2 x 10^4 PC3 cells/well of a 48-well plate using 2 μl of Lipofectamine™ 2000 according to the manufacturer's instructions. 4 h post-transfection the cells were returned to growth media (RPMI 1640 supplemented with 10 % FBS). Viability was assayed by MTT 48 h following transfection.

12) Wound healing migration assay

PC3 cells were seeded into 6-well plates and cultured until confluent. 10 μM AS-10 or sense ODN as control were introduced to the wells as described for the viability assay 12 hours before wounding the monolayer by scraping it with a sterile pipette tip. Medium was changed to RPMI 1640 supplemented with 5% FBS and fresh ODNs. Confluent cultures transfected with 50 nM siRNA 472 or GAPDH negative control siRNA 12 hours prior to wounding were also examined. The healing process was examined dynamically and recorded with a Nikon Coolpix 5000 digital camera with microscope adapter.

13) Invasion assay

PC3 cells were transfected with siRNA 472 or control siRNA using Lipofectamine™ 2000 and 6 hours later 0.5 x 105 cells were transferred into 8 μm Matrigel-precoated inserts (BD Bioscience, Palo Alto, CA). The inserts were placed in companion wells containing RPMI supplemented with 5 % FBS and 5 μg/ml fibronectin as a chemoattractant. Following 22 h incubation the inserts were removed and the noninvading cells on the upper surface were removed by with a cotton swab. The cells on the lower surface of the membrane were fixed in 100% methanol for 15 min, air dried and stained with Giemsa stain for 2 min. The cells were counted in five individual high-powered fields for each membrane under a light microscope. Assays were performed in triplicate for each treatment group.

14) Cell cycle analysis

80% confluent cultures of PC3 cells in 6-well plates were transfected with siRNA472 (100 nM) using Lipofectamine™ 2000. 24 hours after transfection, cells were trypsinized, washed in PBS and incubated for 1 h at 4oC in 1 ml of hypotonic solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1 Triton X-100 and 20 μg/ml Dnase-free RnaseA. Cells were analyzed in linear mode at the USC Flow cytometry facility. Results were expressed as percentages of elements detected in the different phases of the cell cycle, namely Sub G0 peak (apoptosis), G0/G1 (no DNA synthesis), S (active DNA systhesis), G2 (premitosis) and M (mitosis).

15) Apoptosis ELISA

Apoptosis was studied using the Cell Death Detection ELISAplus Kit (Roche, Piscataway, NJ) according to the manufacturer's instructions. Briefly, PC3 80% confluent

cultures in 24-well plates were transfected using Lipofectamine™ 2000 with various concentrations (0-100 nM) of siRNA 472 or 100 nM control siRNA. 16 hours later, cells were detached and 1 x 10^4 cells were incubated in 200 µl lysis buffer. Nuclei were pelleted by centrifugation and 20 µl of supernatant containing the mono- or oligonucleosomes was taken for ELISA analysis. Briefly, the supernatant was incubated with anti-histone-biotin and anti-DNA-POD in streptavidin-coated 96-well plate for 2 hours at room temperature. The color was developed with ABST and absorbance at 405 nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA).

Example 4. Expression of EPHB4 in Mesothelioma: a candidate target for therapy

Malignant mesothelioma (MM) is a rare neoplasm that most often arises from the pleural and peritoneal cavity serous surface. The pleural cavity is by far the most frequent site affected (> 90%), followed by the peritoneum (6-10%) (Carbone et al., 2002, Semin Oncol. 29:2-17). There is a strong association with asbestos exposure, about 80% of malignant mesothelioma cases occur in individuals who have ingested or inhaled asbestos. This tumor is particularly resistant to the current therapies and, up to now, the prognosis of these patients is dramatically poor (Lee et al., 2000, Curr Opin Pulm Med. 6:267-74).

Several clinical problems regarding the diagnosis and treatment of malignant mesothelioma remain unsolved. Making a diagnosis of mesothelioma from pleural or abdominal fluid is notoriously difficult and often requires a thoracoscopic or laproscopic or open biopsy and Immunohistochemical staining for certain markers such as meosthelin expressed preferentially in this tumor. Until now, no intervention has proven to be curative, despite aggressive chemotherapeutic regimens and prolonged radiotherapy. The median survival in most cases is only 12–18 months after diagnosis.

In order to identify new diagnostic markers and targets to be used for novel diagnostic and therapeutic approaches, we assessed the expression of EPHB4 and its ligand EphrinB2 in mesothelioma cell lines and clinical samples.

A. EPHB4 and EphrinB2 is expressed in mesothelioma cell lines

The expression of Ephrin B2 and EphB4 in malignant mesothelioma cell lines was determined at the RNA and protein level by a variety of methods. RT-PCR showed that all of the four cell lines express EphrinB2 and EPHB4 (fig. 33A). Protein expression was determined by Western blot in these cell lines. Specific bands for EphB4 were seen at 120 kD. In addition, Ephrin B2 was detected in all cell lines tested as a 37 kD band on Western blot (fig. 33B). No specific band for Ephrin B2 was observed in 293 human embryonic kidney cells, which were included as a negative control.

To confirm the presence of EphB4 transcription in mesothelioma cells, *in situ* hybridization was carried out on NCI H28 cell lines cultured on chamber slides. Specific signal for EphB4 was detected using antisense probe Ephrin B2 transcripts were also detected in the same cell line. Sense probes for both EphB4 and Ephrin B2 served as negative controls and did not hybridize to the cells (figure 34). Expression of EphB4 and Ephrin B2 proteins was confirmed in the cell lines by immunofluorescence analysis (fig. 35). Three cell lines showed strong expression of EphB4, whereas expression of Ephrin B2 was present in H28 and H2052, and weakly detectable in H2373.

B. Evidence of Expression of EPHB4 and EphrinB2 in clinical samples

Tumor cells cultured from the pleural effusion of a patient diagnosed with pleural malignant mesothelioma were isolated and showed positive staining for both EphB4 and Ephrin B2 at passage 1 (figure 35, bottom row). These results confirm co-expression of EphB4 and Ephrin B2 in mesothelioma cell lines. To determine whether these results seen in tumor cell lines were a real reflection of expression in the disease state, tumor biopsy samples were subjected to immunohistochemical staining for EphB4 and Ephrin B2. Antibodies to both proteins revealed positive stain in the tumor cells. Representative data is shown in figure 36.

C. EPHB4 is involved in the cell growth and migration of mesothelioma

The role of EphB4 in cell proliferation was tested using EPHB4 specific antisepses oligonucleotides and siRNA. The treatment of cultured H28 with EPHB4 antisense reduced cell viability. One of the most active inhibitor of EphB4 expression is EPHB4AS-10 (fig. 37A). Transfection of EPHB4 siRNA 472 generated the same effect (fig. 37B).

MM is a locally advancing disease with frequent extension and growth into adjacent vital structures such as the chest wall, heart, and esophagus. In an effort to study this process in vitro, we perform wound healing assay using previously described techniques (3:36). When a wound was introduced into sub confluent H28 cells, over the course of the next 28 hours cells would progressively migrate into the area of the wound. However, when cells were pretreated with EPHB4AS-10 for 24 hours, and the wound was introduced, this migration was virtually completely prevented (fig. 38A). The migration study with Boyden Chamber assay with EPHB4 siRNA showed that cell migration was greatly inhibited with the inhibition of EPHB4 expression (Fig. 38B).

D. Materials and Methods

1) Cell lines and reagents

NCI H28, NCI H2052, NCI H2373, MSTO 211H mesothelioma cell lines and 293 human embryonic kidney cells were obtained from the ATCC (Manassas, VA). Cells were maintained in RPMI 1640 media supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) and antibiotics. Primary cells were obtained from pleural effusion of patients with mesothelioma. A large number of EPHB4 phosphorothioate modified antisense oligonucleotides were synthesized. Similarly a number of EphB4 specific siRNAs were generated. Monoclonal antibody produced against EPHB4 was used for western blot. Polyclonal antibody against EphrinB2 and EPHB4 (C-16) (for immunohistochemical staining) was from Santa Cruz.

2) RT-PCR

Total RNA was reversed transcribed by use of random hexamers (Invitrogen). Primers for EphB4 and EphrinB2 were designed with Primer 3 software. The sequences for all primers are as follows: EPHB4 forward primer and EPHB4 reverse primer (see, e.g., in Example 2); EphrinB2 forward primer and EphrinB2 reverse primer (see, e.g., in Example 6); G3PDH forward primer, 5'-GGAGCCAAAAGGGTCATCAT-3'; G3PDH reverse primer, 5'-GGCATTGCTGCAAAGAAAGAG-3'; Clonetics kit was used for PCR. PCRs were performed with the ABI PCR System 2700 (Applied Biosystem). The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1min.

3) Preparation of digoxigenin-labeled RNA probes

Ephrin-B2 and EphB4 PCR products were cloned using the pGEM-T Easy System (Promega, Madison WI) according to the manufacturer's description. The primers and PCR products were 5'-tccgtgtggaagtactgctg-3' (forward), 5'-tctggtttggcacagttgag-3' (reverse), for ephrin-B2 that yielded a 296-bp product and 5'-ctttggaagagaccctgctg-3' (forward), 5'-agacggtgaaggtctccttg-3', for EphB4 that yielded a 297-bp product. The authenticity and insert orientation were confirmed by DNA sequencing.

The pGEM-T Easy plasmids containing the PCR product of the human ephrin-B2 or EphB4 gene were linearized with *Spe* I or *Nco* I. Antisense or sense digoxigenin (DIG)-labeled RNA probes were transcribed from T7 or SP6 promoters by run-off transcription using a DIG RNA labeling kit (Roche, Indianapolis IN). RNA probes were quantitated by spot assay as described in the DIG RNA labeling kit instructions.

4) *In situ* hybridization

Cells were cultured in Labtech II 4-well chamber slides (Nalge Nunc International, Naperville, IL). Cells were washed in PBS (37 °C), then fixed for 30 min at 25 °C in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl. After fixation, slides were rinsed with PBS and stored in 70% ethanol at 4 °C until further use. Before *in situ* hybridization, cells were dehydrated, washed in 100% xylene to remove residual lipid and then rehydrated, finally in PBS. Cells were permeabilized by incubating at 37 °C with 0.1% (w/v) pepsin in 0.1 N HCl for 20 min and post-fixed in 1% formaldehyde for 10 min. Prehybridization was performed for 30 min at 37 °C in a solution of 4 X SSC containing 50%(v/v) deionized formamide. Slides were hybridized overnight at 42 °C with 25 ng antisense or sense RNA probes in 40% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 4 X SSC, 10 mM DTT, 1 mg/ml yeast t-RNA and 1mg/ml denatured and sheared salmon sperm DNA in a total volume of 40 µl. Slides were then washed at 37 °C as follows: 2 X 15 min with 2 X SSC, 2 X 15min with 1 X SSC, 2 X 15 min with 0.5 X SSC and 2 X 30 min with 0.2 X SSC. Hybridization signal was detected using alkaline-phosphatase-conjugated anti-DIG antibodies (Roche) according to the manufacturer's instructions. Color development was stopped by two washes in 0.1 M Tris-HCl, 1mM EDTA, pH 8.0 for 10 min. Cells were visualized by counterstaining of nucleic acids with Nuclear Fast Red

(Vector Laboratories, Burlingame, CA) and the slides were mounted with IMMU-MOUNT (Shandon, Astmoor UK).

5) Western Blot

Crude cell lysates were prepared by incubation in cell lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 1 mM DTT, 10 % glycerol). Lysates were cleared by centrifugation at 10,000 x *g* for 10 min. Total protein was determined by Bradford assay (Bio-Rad). Samples (20 μg protein) were fractionated on a 4-20 % Tris-glycine polyacrylamide gel and transferred to polyvinylidene difluoride (PVDT) membrane (Bio-Rad) by electroblotting. Membranes were blocked with 5 % non-fat milk prior to incubation with antibody to EphB4 (1:5000 dilution) at 4° C, for 16 h. Secondary antibody (1:100,000 dilution) conjugated with horseradish peroxidase was applied for 1 h at 25 °C. The membranes were developed using the SuperSignal West Femto Maximum sensitivity chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

6) Immunohistochemistry

Formalin-fixed tissue sections were deparaffinized and incubated with 10% goat serum at -70 °C for 10 minutes and incubated with the primary rabbit antibodies against either Ephrin B2 or EphB4 (Santa Cruz Biotechnologies; 1:100) at 4 °C overnight. Isotype-specific rabbit IgG was used as control. The immunoreactivity for these receptors was revealed using an avidin-biotin kit from Vector Laboratories. Peroxidase activity was revealed by the diaminobenzidine (Sigma) cytochemical reaction. The slides were then counterstained with H&E.

7) Immunofluorescence studies

Cells were cultured on Labtech II 4-well chamber slides and fixed in 4% paraformaldehyde in Dulbecco's phosphate buffered saline pH 7.4 (PBS) for 30 min. The slides were rinsed twice in PBS and preincubated with blocking buffer (0.2% Triton-X100, 1% BSA in PBS) for 20 min. The slides were then incubated with antibodies to EphB4 or ephrin B2 (1:100 dilution in PBS) in blocking buffer at 4 °C for 16 hr. After washing three times, the slides were incubated with the appropriate fluorescein-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), washed extensively with PBS and mounted with Vectasheild antifade mounting

solution (Vector Laboratories). Images were obtained using an Olympus AX70 fluorescence microscope and Spot v2.2.2 (Diagnostic Instruments Inc., Sterling Heights, MI) digital imaging system.

8) Cell viability assay

Cells were seeded at a density of 5 x 10^3 per well in 48-well plates on day 0 in appropriate growth media containing 2% fetal calf serum (FCS). On the following day, the media was changed and cells were treated with various concentrations (1-10 μM) of EphB4 Antisense. On day 4, viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml. Cells were incubated for 2 hr, medium was aspirated, and the cells were dissolved in acidic isopropanol (90% isopropanol, 0.5% SDS and 40 mM HCl). Optical density was read in an ELISA reader at 490 nm using isopropanol as blank (Molecular Devices, CA).

9) Cell migration

In vitro wound healing assay was adopted. Briefly, cells were seeded onto 6-cm plates in full culture media for 24 hours, and then switched to medium containing 5% FBS. EPHB4 antisense 10 (10 μM) was also added to treated well. 24 hours later, wounds were made using the tip of a p-200 pipette man; a line was drawn through the middle of the plates. The plate was photographed at 0, 12, 24 hours. The experiment was repeated three times.

Example 5. EphB4 Is Expressed in Squamous Cell Carcinoma of The Head and Neck: Regulation by Epidermal Growth Factor Signaling Pathway and Growth Advantage.

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent cancer worldwide, with estimated 900,000 cases diagnosed each year. It comprises almost 50% of all malignancies in some developing nations. In the United States, 50,000 new cases and 8,000 deaths are reported each year. Tobacco carcinogens are believed to be the primary etiologic agents of the disease, with alcohol consumption, age, gender, and ethnic background as contributing factors.

The differences between normal epithelium of the upper aerodigestive tract and cancer cells arising from that tissue are the result of mutations in specific genes and alteration of their expression. These genes control DNA repair, proliferation, immortalization, apoptosis, invasion,

and angiogenesis. For head and neck cancer, alterations of three signaling pathways occur with sufficient frequency and produce such dramatic phenotypic changes as to be considered the critical transforming events of the disease. These changes include mutation of the p53 tumor suppressor, overexpression of epidermal growth factor receptor (EGFR), and inactivation of the cyclin dependent kinase inhibitor p16. Other changes such as Rb mutation, ras activation, cyclin D amplification, and myc overexpression are less frequent in HNSCC.

Although high expression of EphB4 has been reported in hematologic malignancies, breast carcinoma, endometrial carcinoma, and colon carcinoma, there is limited data on the protein levels of EphB4, and complete lack of data on the biological significance of this protein in tumor biology such as HNSCC.

A. HNSCC tumors express EphB4

We studied the expression of EphB4 in human tumor tissues by immunohistochemistry, in situ hybridization, and Western blot. Twenty prospectively collected tumor tissues following IRB approval have been evaluated with specific EphB4 monoclonal antibody that does not react with other members of the EphB and EphA family. EphB4 expression is observed in all cases, with varying intensity of staining. Figure 39A (top left) illustrates a representative case, showing that EphB4 is expressed in the tumor regions only, as revealed by the H&E tumor architecture (Fig. 39A bottom left). Note the absence of staining for EphB4 in the stroma. Secondly, a metastatic tumor site in the lymph node shows positive staining while the remainder of the lymph node is negative (Fig. 39A, top right).

In situ hybridization was carried out to determine the presence and location of EphB4 transcripts in the tumor tissue. Strong signal for EphB4 specific antisense probe was detected indicating the presence of transcripts (Figure 39 B, top left). Comparison with the H&E stain (Fig. 39B, bottom left) to illustrate tumor architecture reveals that the signal was localized to the tumor cells, and was absent from the stromal areas. Ephrin B2 transcripts were also detected in tumor sample, and as with EphB4, the signal was localized to the tumor cells (Fig. 39B, top right). Neither EphB4 nor ephrin B2 sense probes hybridized to the sections, proving specificity of the signals.

B. High expression of EphB4 in primary and metastatic sites of HNSCC

Western blots of tissue from primary tumor, lymph node metastases and uninvolved tissue were carried out to determine the relative levels of EphB4 expression in these sites. Tumor and normal adjacent tissues were collected on 20 cases, while lymph nodes positive for tumor were harvested in 9 of these 20 cases. Representative cases are shown in figure 39C. EphB4 expression is observed in each of the tumor samples. Similarly, all tumor positive lymph nodes show EphB4 expression that was equal to or greater than the primary tumor. No or minimal expression is observed in the normal adjacent tissue.

C. EphB4 expression and regulation by EGFR activity in HNSCC cell lines

Having demonstrated the expression of EphB4 limited to tumor cells, we next sought to determine whether there was an in vitro model of EphB4 expression in HNSCC. Six HN SCC cell lines were surveyed for EphB4 protein expression by Western Blot (Fig. 40A). A majority of these showed strong EphB4 expression and thus established the basis for subsequent studies. Since EGFR is strongly implicated in HNSCC we asked whether EphB4 expression is associated with the activation of EGFR. Pilot experiments in SCC-15, which is an EGFR positive cell line, established an optimal time of 24 h and concentration of 1 mM of the specific EGFR kinase inhibitor AG 1478 (Figure 40B) to inhibit expression of EphB4. When all the cell lines were studied, we noted robust EGFR expression in all but SCC-4, where it is detectable but not strong (Fig. 40C, top row). In response to EGFR inhibitor AG1478 marked loss in the total amount of EphB4 was observed in certain cell lines (SCC-15, and SCC-25) while no effect was observed in others (SCC-9, -12, -13 and -71). Thus SCC-15 and -25 serve as models for EphB4 being regulated by EGFR activity, while SCC-9, -12, -13 and -71 are models for regulation of EphB4 in HNSCC independent of EGFR activity, where there may be input from other factors such as p53, PTEN, IL-6 etc. We also noted expression of the ligand of EphB4, namely ephrin B2, in all of the cell lines tested. As with EphB4 in some lines ephrin B2 expression appears regulated by EGFR activity, while it is independent in other cell lines.

Clearly, inhibition of constitutive EGFR signaling repressed EphB4 levels in SCC15 cells. We next studied whether EGF could induce EphB4. We found that EphB4 levels were induced in SCC15 cells that had been serum starved for 24 h prior to 24 h treatment with 10 ng/ml EGF as shown in figure 41B (lanes 1 and 2). The downstream signaling pathways known for EGFR activation shown in figure 41A, (for review see Yarden & Slikowski 2001) were then

investigated for their input into EGF mediated induction of EphB4. Blocking PLCg, AKT and JNK phosphorylation with the specific kinase inhibitors U73122, SH-5 and SP600125 respectively reduced basal levels and blocked EGF stimulated induction of EphB4 (Fig. 41B, lanes 3-8). In contrast, inhibition of ERK1/2 with PD098095 and PI3-K with LY294002 or Wortmannin had no discernible effect on EGF induction of EphB4 levels. However, basal levels of EphB4 were reduced when ERK1/2 phosphorylation was inhibited. Interestingly, inhibition of p38 MAPK activation with SB203580 increased basal, but not EGF induced EphB4 levels. Similar results were seen in the SCC25 cell line (data not shown).

D. Inhibition of EphB4 in high expressing cell lines results in reduced viability and causes cell-cycle arrest

We next turned to the role of EphB4 expression in HNSCC by investigating the effect of ablating expression using siRNA or AS-ODN methods. Several siRNAs to EphB4 sequence were developed (Table 1) which knocked-down EphB4 expression to varying degrees as seen in figure 42A. Viability was reduced in SCC-15, -25 and -71 cell lines transfected with siRNAs 50 and 472, which were most effective in blocking EphB4 expression (Figure 42B). Little effect on viability was seen with EphB4 siRNA 1562 and 2302 or ephrin B2 siRNA 254. Note that in SCC-4, which does not express EphB4 (see Fig. 40A) there was no reduction in cell viability. The decreased cell viability seen with siRNA 50 and 472 treatment was attributable to accumulation of cells in sub G0, indicative of apoptosis. This effect was both time and dose-dependant (Figure 42C and Table 2). In contrast, siRNA2302 that was not effective in reducing EphB4 levels and had only minor effects on viability did not produce any changes in the cell cycle when compared with the mock Lipofectamine™2000 transfection.

Table 1: EphB4 siRNAs

Name	siRNA sequence
Eph B4 50:	5′-GAGACCCUGCUGAACACAAUU-3′ 3′-UUCUCUGGGACGACUUGUGUU-5′
Eph B4 472:	5′-GGUGAAUGUCAAGACGCUGUU-3′ 3′-UUCCACUUACAGUUCUGCGAC-5′
Eph B4 1562:	5′-CAUCACAGCCAGACCCAACUU-3′ 3′-UUGUAGUGUCGGUCUGGGUUG-5′

Eph B4 2302	5′-CUCUUCCGAUCCCACCUACUU-3′ 3′-UUGAGAAGGCUAGGGUGGAUG-5′

Table 2: Effect of different EphB4 siRNA on Cell Cycle

Treatment	Sub G0	G1	S	G2
36hr				
Lipo alone	1.9	39.7	21.3	31.8
100 nM 2302	2.0	39.3	21.2	31.2
100 nM 50	18.1	31.7	19.7	24.4
100 nM 472	80.2	10.9	5.2	2.1
16hr				
Lipo alone	7.8	55.7	15.2	18.5
100 nM 2302	8.4	57.3	14.3	17.3
10 nM 50	10.4	53.2	15.7	17.7
100 nM 50	27.7	31.3	18.1	19.6
10 nM 472	13.3	50.2	15.8	17.5
100 nM 472	30.7	31.9	16.4	18.0

In addition, over 50 phosphorothioate AS-ODNs complementary to the human EphB4 coding sequences were synthesized and tested for their ability to inhibit EphB4 expression in 293 cells transiently transfected with full length EphB4 expression plasmid. Figure 43A shows a representative sample of the effect of some of these AS-ODNs on EphB4 expression. Note that expression is totally abrogated with AS-10, while AS-11 has only a minor effect. The effect on cell viability in SCC15 cells was most marked with AS-ODNs that are most effective in inhibiting EphB4 expression as shown in figure 43B. The IC_{50} for AS-10 was approximately 1 μM, while even 10 μM AS-11 was not sufficient to attain 50 % reduction of viability. When the

effect that AS-10 had on the cell cycle was investigated, it was found that the sub G0 fraction increased from 1.9 % to 10.5 % compared to non-treated cells, indicative of apoptosis (Fig. 43C).

E. EphB4 regulates Cell migration

We next wished to determine if EphB4 participates in the migration of HNSCC. Involvement in migration may have implications for growth and metastasis. Migration was assessed using the wound-healing/scrape assay. Confluent SCC15 and SCC25 cultures were wounded by a single scrape with a sterile plastic Pasteur pipette, which left a 3 mm band with clearly defined borders. Migration of cells into the cleared area in the presence of test compounds was evaluated and quantitated after 24, 48 and 72 hr. Cell migration was markedly diminished in response to AS-10 that block EphB4 expression while the inactive compounds, AS-1 and scrambled ODN had little to no effect as shown in figure 43D. Inhibition of migration with AS-10 was also shown using the Boyden double chamber assay (Fig. 43E).

F. EphB4 AS-10 in vivo anti-tumor activity

The effect of EphB4 AS-10, which reduces cell viability and motility, was determined in SCC15 tumor xenografts in Balb/C nude mice. Daily treatment of mice with 20 mg/kg AS-10, sense ODN or equal volume of PBS by I.P. injection was started the day following tumor cell implantation. Growth of tumors in mice receiving AS-10 was significantly retarded compared to mice receiving either sense ODN or PBS diluent alone (Figure 44). Non-specific effects attributable to ODN were not observed, as there was no difference between the sense ODN treated and PBS treated groups.

G. Materials and Methods

1) Cell lines and reagents

HNSCC-4, -9, 12, -13, -15, -25, and -71 were obtained from and 293 human embryonic kidney cells were obtained from the ATCC (Manassas, VA). Cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and antibiotics. EGFR, EphB4(C-16) polyclonal antibodies were from Santa Cruz Biotech (Santa Cruz, CA). β-actin monoclonal antibody was purchased from Sigma Chemical Co. (St Louis, MO). Ephrin B2 and EphB4 polyclonal antibodies and their corresponding blocking peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). AG 1478 (4-

(3'-Chloroanilino)-6,7-dimethoxy-quinazoline) was from Calbiochem (San Diego, CA). Kinase inhibitors SH-5 and SP 600125 were from A.G. Scientific (San Diego, CA), PD98095, U73122, SB203580, LY294002, and Wortmannin were obtained from Sigma.

2) Preparation of digoxigenin-labeled RNA probes

See above, e.g., Example 3.

3) *In situ* hybridization

See above, e.g., Example 3.

4) Immunohistochemistry

Formalin-fixed tissue sections were deparaffinized and incubated with 10% goat serum at -70 °C for 10 minutes and incubated with the EphB4 monoclonal antibody 4 °C overnight. Isotype specific rabbit IgG was used as control. The immunoreactivity for these receptors was revealed using an avidin-biotin kit from Vector Laboratories. Peroxidase activity was revealed by the diaminobenzidine (Sigma) cytochemical reaction. The slides were then counterstained with 0.12% methylene blue or H&E. For frozen sections, OCT-embedded tissues were sectioned at 5 μm and fixed in phosphate-buffered 4% paraformaldehyde. Sections were washed for 3 x 5 min in PBS and endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in PBS for 10 min at room temperature. Sections were incubated with Eph4 (C-16) antibody (1:50) for 1 h at room temperature followed by three washes in PBS and incubation with donkey anti-goat secondary antibody (Santa Cruz Biotech.) for 1 h at room temperature. After three washes in PBS, peroxidase activity was localized by incubation in DAB substrate solution (Vector Laboratories, Inc. Burlingame CA) for 10 min at room temperature. Sections were counterstained with Hematoxylin for 20 s, dehydrated and mounted. Negative control for staining was substitution of normal goat serum for primary antibody. Immunohistochemical staining on prostate array (BioMeda, Foster City, CA) was done using goat ABC Staining System (Santa Cruz Biotech.) according to the manufacturer's instructions.

5) Western Blot

See above, e.g., Example 3.

6) Synthesis of EphB4 siRNA by in vitro transcription

The Silencer™ siRNA construction kit (Ambion, Austin TX) was used to synthesize siRNA to EphB4. Briefly, 21 bp target sequences containing 19 bp downstream of 5'-AA dinucleotides were identified that showed no significant homology to other sequences in the GenBank database. Sense and antisense siRNA 29-mer DNA oligonucleotide templates were synthesized at the USC Norris Microchemical Core Facility. Antisense template corresponded to the target sequence followed by 8 bp addition (5'-CCTGTCTC-3') at the 3' end complementary to the T7 promoter primer provided by the Silencer™ siRNA construction kit. Sense template comprised 5'-AA followed by the complement of the target 19 bp, then the T7 8 bp sequence as above.

In separate reactions, the two siRNA oligonucleotide templates were hybridized to a T7 promoter primer. The 3' ends of the hybridized oligonucleotides were extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The leader sequences were removed by digesting the dsRNA with a single-stranded specific ribonuclease leaving the overhanging UU dinucleotides. The DNA template was removed at the same time by treatment with RNase free deoxyribonuclease. The resulting siRNA was purified by glass fiber filter binding to remove excess nucleotides, short oligomers, proteins, and salts in the reaction. The end products (shown in Table 3) were double-stranded 21-mer siRNAs with 3' terminal uridine that can effectively reduce the expression of target mRNA when transfected into cells.

A number of phosphorothioate AS-ODNs were also synthesized (Operon, Valencia CA) to test for inhibition of EphB4 expression (Table 3).

Table 3: EphB4 Antisense ODNs

Name	Position	Sequence (5' → 3')
Eph B4 AS-1	(552-572)	GTG CAG GGA TAG CAG GGC CAT
Eph B4 AS-2	(952-972)	AAG GAG GGG TGG TGC ACG GTG
Eph B4 AS-3	(1007-1027)	TTC CAG GTG CAG GGA GGA GCC
Eph B4 AS-4	(1263-1285)	GTG GTG ACA TTG ACA GGC TCA

Eph B4 AS-5	(1555-1575)	TCT GGC TGT GAT GTT CCT GGC
Eph B4 AS-6	(123-140)	GCC GCT CAG TTC CTC CCA
Eph B4 AS-7	(316-333)	TGA AGG TCT CCT TGC AGG
Eph B4 AS-8	(408-428)	CGC GGC CAC CGT GTC CAC CTT
Eph B4 AS-9	(1929-1949)	CTT CAG GGT CTT GAT TGC CAC
Eph B4 AS-10	(1980-1999)	ATG GAG GCC TCG CTC AGA AA
Eph b4 AS-11	(2138-2158)	CAT GCC CAC GAG CTG GAT GAC

7) Cell viability assay

Cells were seeded at a density of 5 x 10^3 per well in 48-well plates on day 0 in appropriate growth media containing 2% fetal calf serum (FCS). Cells were treated with various concentrations (1-10 μg/ml) of ODNs on days 2 and 4. On day 5, viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (Masood et al '03). For viability with siRNA, 2 x 10^4 cells/well of SCC-4, -15, -25 or -71 in a 48-well plate were transfected with siRNAs (10-100 nM) using 2 μl of Lipofectamine™ 2000 according to the manufacturer's instructions. 4 h post-transfection the cells were returned to growth media (RPMI 1640 supplemented with 10 % FBS). Viability was assayed by MTT 48 h following transfection.

8) Cell cycle analysis

80% confluent cultures of SCC15 cells in 6-well plates were transfected with siRNA472 (100 nM) using Lipofectamine™ 2000. Either 16 or 36 hours after transfection, cells were trypsinized, washed in PBS and incubated for 1 h at 4 °C in 1 ml of hypotonic solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1 Triton X-100 and 20 μg/ml DNase-free RNaseA. Cells were analyzed in linear mode at the USC Flow cytometry facility. Results were expressed as percentages of elements detected in the different phases of the cell cycle, namely Sub G0 peak (apoptosis), G0/G1 (no DNA synthesis), S (active DNA systhesis), G2 (premitosis) and M (mitosis). For AS-ODN experiment the cells were exposed to 5 μM ODN for 36 h prior to processing.

9) Wound healing migration assay

SCC15 cells were seeded into 6-well plates and cultured until confluent. 10 μM AS-1, AS-10, or sense ODN as control were introduced to the wells as described for the viability assay 12 hours before wounding the monolayer by scraping it with a sterile pipette tip. Medium was changed to RPMI 1640 supplemented with 5% FBS and fresh ODNs. The healing process was examined dynamically and recorded with a Nikon Coolpix 5000 digital camera with microscope adapter.

10) Boyden Chamber assay of migration

Cell migration assays were performed as previously described (Masood ANUP paper '99) except that 1 μM AS-10 or AS-6 were added to the upper chamber. EGF (20 ng/ml) was used as chemoattractant in the lower chamber. Taxol at 10 ng/ml was used as a negative control.

11) In vivo studies

SCC15 (5×10^6 cells) were injected subcutaneously in the lower back of 5-week old male Balb/C Nu^+/nu^+ athymic mice. Treatment consisted of daily intraperitoneal injection of ODN (20 mg/kg in a total volume of 100 μl) or diluent (PBS) begun the day following tumor cell implantation and continued for two weeks. Tumor growth in mice was measured as previously described (Masood CCR '01). Mice were sacrificed at the conclusion of the study. All mice were maintained in accord with the University of Southern California Animal Care and Use Committee guidelines governing the care of laboratory mice.

Example 6. Ephrin B2 Expression in Kaposi's Sarcoma Is Induced by Human Herpesvirus Type 8: Phenotype Switch from Venous to Arterial Endothelium

Kaposi's Sarcoma (KS) manifests as a multifocal angioproliferative disease, most commonly of the skin and mucus membranes, with subsequent spread to visceral organs (1) Hallmarks of the disease are angiogenesis, edema, infiltration of lymphomononuclear cells and growth of spindle-shaped tumor cells. Pathologically, established lesions exhibit an extensive vascular network of slit-like spaces. The KS vascular network is distinct from normal vessels in the lack of basement membranes and the abnormal spindle shaped endothelial cell (tumor cell) lining these vessels. Defective vasculature results in an accumulation of the blood components

including albumin, red and mononuclear cells in the lesions (1). The KS tumor is endothelial in origin; the tumor cells express many endothelial markers, including lectin binding sites for *Ulex europeaus* agglutinin-1 (UEA-1), CD34, EN-4, PAL-E (2) and the endothelial cell specific tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), VEGFR-3 (Flt-4), Tie-1 and Tie-2 (3, RM & PSG unpublished data). KS cells co-express lymphatic endothelial cell related proteins including LYVE and podoplanin (4).

The herpesvirus HHV-8 is considered the etiologic agent for the disease. In 1994 sequences of this new herpes virus were identified in KS tumor tissue (5), and subsequent molecular-epidemiology studies have shown that nearly all KS tumors contain viral genome. Sero-epidemiology studies show that HIV infected patients with KS have the highest prevalence of HHV-8 and secondly that those with HIV infection but no KS have increased risk of developement of KS over the ensuing years if they are also seropositive for HHV-8 (6). Direct evidence for the role of HHV-8 in KS is the transformation of bone marrow endothelial cells after infection with HHV-8 (7). A number of HHV-8 encoded genes could contribute to cellular transformation (reviewed in 8). However, the most evidence has accumulated for the G-protein coupled receptor (vGPCR) in this role (9).

We investigated whether KS tumor cells are derived from arterial or venous endothelium. In addition, we investigated whether HHV-8 has an effect on expression of arterial or venous markers in a model of KS. KS tumor cells were found to express the ephrin B2 arterial marker. Further, ephrin B2 expression was induced by HHV-8 vGPCR in KS and endothelial cell lines. Ephrin B2 is a potential target for treatment of KS because inhibition of ephrin B2 expression or signaling was detrimental to KS cell viability and function.

A. KS tumors express Ephrin B2, but not EphB4

The highly vascular nature of KS lesions and the probable endothelial cell origin of the tumor cells prompted investigation of expression of EphB4 and ephrin B2 which are markers for venous and arterial endothelial cells, respectively. Ephrin B2, but not EphB4 transcripts were detected in tumor cells of KS biopsies by in situ hybridization (figure 45A). Comparison of the positive signal with ephrin B2 antisense probe and tumor cells as shown by H&E staining shows that ephrin B2 expression is limited to the areas of the biopsy that contain tumor cells. The lack

of signal in KS with EphB4 antisense probe is not due to a defect in the probe, as it detected transcripts in squamous cell carcinoma, which we have shown expresses this protein (18). Additional evidence for the expression of ephrin B2 in KS tumor tissue is afforded by the localization of EphB4/Fc signal to tumor cells, detected by FITC conjugated anti human Fc antibody. Because ephrin B2 is the only ligand for EphB4 this reagent is specific for the expression of ephrin B2 (figure 45B, left). An adjacent section treated only with the secondary reagent shows no specific signal. Two-color confocal microscopy demonstrated the presence of the HHV-8 latency protein, LANA1 in the ephrin B2 positive cells (Fig. 45C, left), indicating that it is the tumor cells, not tumor vessels, which are expressing this arterial marker. Staining of tumor biopsy with PECAM-1 antibody revealed the highly vascular nature of this tumor (Fig. 45C, right). A pilot study of the prevalence of this pattern of ephrin B2 and EphB4 expression on KS biopsies was conducted by RT-PCR analysis. All six samples were positive for ephrin B2, while only 2 were weakly positive for EphB4 (data not shown).

B. Infection of venous endothelial cells with HHV-8 causes a phenotype switch to arterial markers

We next asked whether HHV-8, the presumed etiologic agent for KS, could itself induce expression of ephrin B2 and repress EphB4 expression in endothelial cells. Co-culture of HUVEC and BC-1 lymphoma cells, which are productively infected with HHV-8, results in effective infection of the endothelial cells (16). The attached monolayers of endothelial cells remaining after extensive washing were examined for ephrin B2 and EphB4 by RT-PCR and immunofluorescence. HUVEC express EphB4 venous marker strongly at the RNA level, but not ephrin B2 (figure 46B). In contrast, HHV-8 infected cultures (HUVEC/BC-1 and HUVEC/BC-3) express ephrin B2, while EphB4 transcripts are almost absent.

Immunofluorescence analysis of cultures of HUVEC and HUVEC/HHV-8 for artery/vein markers and viral proteins was undertaken to determine whether changes in protein expression mirrored that seen in the RNA. In addition, cellular localization of the proteins could be determined. Consistent with the RT-PCR data HUVEC are ephrin B2 negative and EphB4 positive (Fig. 46A(a & m)). As expected they do not express any HHV-8 latency associated nuclear antigen (LANA1) (Fig. 46A(b, n)). Co-culture of BC-1 cells, which are productively

infected with HHV-8, resulted in infection of HUVEC as shown by presence of viral proteins LANA1 and ORF59 (Fig. 46A(f, r)). HHV-8 infected HUVEC now express ephrin B2 but not EphB4 (Fig. 46A(e, q, u), respectively). Expression of ephrin B2 and LANA1 co-cluster as shown by yellow signal in the merged image (Fig. 46A(h)). HHV-8 infected HUVEC positive for ephrin B2 and negative for Eph B4 also express the arterial marker CD148 (19) (Fig. 46A (j, v)). Expression of ephrin B2 and CD148 co-cluster as shown by yellow signal in the merged image (Fig. 46A(l)). Uninfected HUVEC expressing Eph B4 were negative for CD148 (not shown).

C. HHV-8 vGPCR induces ephrin B2 expression

To test whether individual viral proteins could induce the expression of ephrin B2 seen with the whole virus KS-SLK cells were stably transfected with HHV-8 LANA, or LANAΔ440 or vGPCR. Western Blot of stable clones revealed a five-fold induction of ephrin B2 in KS-SLK transfected with vGPCR compared to SLK-LANA or SLK-LANAΔ440 (Fig. 47A). SLK transfected with vector alone (pCEFL) was used as a control. SLK-vGPCR and SLK-pCEFL cells were also examined for ephrin B2 and Eph B4 expression by immunofluorescence in transiently transfected KS-SLK cells. Figure 47B shows higher expression of ephrin B2 in the SLK-vGPCR cells compared to SLK-pCEFL. No changes in Eph B4 were observed in SLK-vGPCR compared to SLK-pCEFL. This clearly demonstrates that SLK-vGPCR cells expressed high levels of ephrin B2 compared to SLK-pCEFL cells. This suggests that vGPCR of HHV-8 is directly involved in the induction of Ephrin B2 and the arterial phenotype switch in KS. Since we had shown that HHV-8 induced expression of ephrin B2 in HUVEC, we next asked if this could be mediated by a transcriptional effect. Ephrin B2 5'-flanking DNA-luciferase reporter plasmids were constructed as described in the Materials and Methods and transiently transfected into HUVECs. Ephrin B2 5'-flanking DNA sequences -2491/-11 have minimal activity in HUVEC cells (figure 47C). This is consistent with ephrin B2 being an arterial, not venous marker. However, we have noted that HUVEC in culture do express some ephrin B2 at the RNA level. Cotransfection of HHV-8 vGPCR induces ephrin B2 transcription approximately 10-fold compared to the control expression vector pCEFL. Roughly equal induction was seen with ephrin B2 sequences -2491/-11, -1242/-11, or -577/-11, which indicates that elements between -

577 and -11 are sufficient to mediate the response to vGPCR, although maximal activity is seen with the -1242/-11 luciferase construct.

D. Expression of Ephrin B2 is regulated by VEGF and VEGF-C

We next asked whether known KS growth factors could be involved in the vGPCR-mediated induction of ephrin B2 expression. SLK-vGPCR cells were treated with neutralizing antibodies to oncostatin-M, IL-6, IL-8, VEGF or VEGF-C for 36 hr. Figure 48A shows that neutralization of VEGF completely blocked expression of ephrin B2 in SLK-vGPCR cells. A lesser, but significant decrease in ephrin B2 was seen neutralization of VEGF-C and IL-8. No appreciable effect was seen with neutralization of oncostatin-M or IL-6. To verify that VEGF and VEGF-C are integral to the induction of ephrin B2 expression we treated HUVEC with VEGF, VEGF-C or EGF. HUVECs were grown in EBM-2 media containing 5 % FBS with two different concentration of individual growth factor (10 ng, 100 ng/ml) for 48 h. Only VEGF-A or VEGF-C induced ephrin B2 expression in a dose dependent manner (Figure 48B). In contrast, EGF had no effect on expression of ephrin B2.

E. Ephrin B2 siRNA inhibits the expression of Ephrin B2 in KS

Three ephrin B2 siRNA were synthesized as described in the methods section. KS-SLK cells were transfected with siRNA and 48 h later ephrin B2 expression was determined by Western Blot. Ephrin B2 siRNAs 137 or 254 inhibited about 70% of ephrin B2 expression compared to control siRNA such as siRNA Eph B4 50 or siRNA GFP. Ephrin B2 63 siRNA was less effective than the above two siRNA Ephrin B2 (Figure 49A).

F. Ephrin B2 is necessary for full KS and EC viability, cord formation and in vivo angiogenesis activities

The most effective ephrin B2 siRNA (254) was then used to determine whether inhibiting expression of ephrin B2 has any effect on the growth of KS-SLK or HUVEC cells. The viability of KS-SLK cells was decreased by the same siRNAs that inhibited ephrin B2 protein levels (figure 49B). KS-SLK express high levels of ephrin B2 and this result shows maintenance of ephrin B2 expression is integral to cell viability in this setting. HUVECs do not express ephrin B2, except when stimulated by VEGF as shown in Fig. 48B. Ephrin B2 siRNA 264 dramatically

reduced growth of HUVECs cultured with VEGF as the sole growth factor. In contrast, no significant effect was seen when HUVECs were cultured with IGF, EGF and bFGF. As a control, EphB4 siRNA 50 had no detrimental effect on HUVECs in either culture condition (figure 49C).In addition to inhibition of viability of KS and primary endothelial cells, EphB4-ECD inhibits cord formation in HUVEC and KS-SLK and in vivo angiogenesis in the Matrigel™ plug assay (Figure 50).

G. Methods and Materials

1) Cell lines and reagents

Human vascular endothelial cells (HUVEC) were from Clonetics (San Diego, CA) and were maintained in EGM-2 and EGM-2MV media respectively (Clonetics). T1 human fibroblast line was from Dr. Peter Jones, USC. BC-1 and BC-3 human pleural effusion lymphoma cell lines and monoclonal antibodies to LANA1 and ORF59 were the kind gift of Dr. Dharam Ablashi (Advanced Biotechnologies Inc., Columbia, MD). KS-SLK was isolated from a Classic Kaposi's sarcoma patient (15). Polyclonal antibodies to EphB4, ephrin B2, CD148, PECAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse EphB4/F_c' and monoclonal antibodies to human vascular endothelial growth factor (VEGF), VEGF-C, interleukin-(IL)6, IL-8 and oncostatin-M were purchased from R & D Systems (Minneapolis, MN). Expression vectors pKSvGPCR-CEFL and pCEFL were the kind gift of Dr. Enrique Mesri (Cornell University, New York, NY). Expression vectors for HHV-8 latency associated nuclear antigen (LANA) were kindly provided by Dr Matthew Rettig, Veteran's Administration Greater Los Angeles Healthcare System.

2) Collection and preparation of human tissue

Human cutaneous Kaposi's sarcoma biopsy material was obtained under local anesthesia with informed consent from patients at the LAC/USC Medical Center, using an IRB approved consent form. Biopsies were processed for either total RNA, paraffin blocks or frozen tissue blocks in OCT. Total RNA was extracted by homogenization in guanidine isothiocyanate, (RNAzol: Tel-Test, Inc., Friendswoods, TX). cDNAs were synthesized by reverse transcriptase using a random hexamer primer (Superscript II; Invitrogen, Carlsbad, CA).

3) Preparation of digoxigenin-labeled RNA probes

Ephrin B2 and EphB4 PCR products from the primers shown in Table 4 for in situ hybridization were cloned using the pGEM-T Easy system (Promega, Madison WI) according to the manufacturer's description using. The authenticity and insert orientation were confirmed by DNA sequencing. The pGEM-T Easy plasmids containing the PCR product of the human ephrin-B2 or EphB4 gene were linearized with *Spe* I or *Nco* I. Antisense or sense digoxigenin (DIG)-labeled RNA probes were transcribed from T7 or SP6 promoters by run-off transcription using a DIG RNA labeling kit (Roche, Indianapolis IN). RNA probes were quantitated by spot assay as described in the DIG RNA labeling kit instructions.

Table 4: Primers for Ephrin B2 and EphB4.

Gene	Primer sequence	Product Size (bp)
ISH Probe Primers		
ephrin B2	5'-TCC GTG TGG AGT ACT GCT G-3'	296
	5'-TCT GGT TTG GCA CAG TTG AG-3'	
EphB4	5'-CTT TGG AAG AGA CCC TGC TG-3'	297
	5'-AGA CGG TGA AGG TCT CCT TG-3'	
RT-PCR Primers		
ephrin B2	5'-AGA CAA GAG CCA TGA AGA TC-3'	200
	5'-GGA TCC CAC TTC GGA CCC GAG-3'	
EphB4	5'-TCA GGT CAC TGC ATT GAA CGG G-3'	400
	5'-AAC TCG CTC TCA TCC AGT T-3'	
β-actin	5'-GTG GGG CGC CCC AGG CAC CA-3'	546
	5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'	

4) *In situ* hybridization

See above, e.g., Example 3.

5) Co-culture of HUVEC and BC-1

HUVEC cells were grown to 50-70% confluence in EGM-2 on gelatin-coated Labtech II 4-well chamber slides (Nalge Nunc International, Naperville, IL). Co-culture with BC-1 or BC-3 was essentially as described by Sakurada and coworkers (16). Briefly, BC-1 or BC-3 cells were pretreated with TPA (20 ng/ml) to induce virus for 48 hrs and then added to the HUVEC culture at a ratio of 10:1 for cocultivation for two days. The HUVECs were washed extensively with PBS to remove the attached BC-1 or BC-3 cells.

6) Preparation of cDNA and RT-PCR

The TITANIUM™ One-Step RT-PCR kit (Clontech, Palo Alto, CA) was used for RT-PCR from 1 x 10^5 cells. Primer pairs for amplification of EphB4, ephrin B2 and β-actin are shown in Table 4. Each PCR cycle consisted of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The samples were amplified for 30 cycles. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

7) Cell viability assay

KS-SLK cells were seeded at a density of 1 x 10^4 per well in 48-well plates on day 0 in appropriate growth media containing 2% fetal calf serum (FCS). On the following day, the media was changed and cells were treated with 0, 10 or 100 nM siRNA. On day 3, viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (17).

8) Immunofluorescence studies

Cells cultured on Labtech II 4-well chamber slides or frozen sections of KS biopsy material were fixed in 4% paraformaldehyde in Dulbecco's phosphate buffered saline pH 7.4 (PBS) for 30 min. The slides were rinsed twice in PBS and preincubated with blocking buffer (0.2% Triton-X100, 1% BSA in PBS) for 20 min, followed by incubation with antibodies to EphB4, ephrin B2, CD148, LANA1 or ORF59 (1:100 dilution in PBS) in blocking buffer at 4 °C for 16 hr. After washing three times, the slides were incubated with the appropriate fluorescein or rhodamine-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Nuclei were

counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), washed extensively with PBS and mounted with Vectasheild antifade mounting solution (Vector Laboratories, Burlingame, CA). Images were obtained using a Olympus AX70 fluorescence microscope and Spot v2.2.2 (Diagnostic Instruments Inc., Sterling Heights, MI) digital imaging system.

Immunofluorescence detection of EphrinB2 with EPHB4-Fc was done as follows. Frozen sections fixed in 4% paraformaldehyde and blocked with 20% FBS were incubated with 5 μg/ml EphB4/Fc (R&D Systems) for 1 h at RT. Sections were then incubated with 10 μg/ml rabbit anti-human IgG-FITC in PBS (Jackson ImmunoResearch Laboratories West Grove, PA) at RT for 1 hour. Nuclei were counterstained with DAPI and sections mounted as above. Human Fc (Jackson ImmunoResearch) was used as the negative control.

9) Western Blot

Crude cell lysates were prepared, quantitated, fractionated and transferred to membranes as described previously (17). Membranes were blocked with 5% non-fat milk prior to incubation with antibody to ephrin B2 (1:5000 dilution) at 4 °C, for 16 h. Secondary antibody (1:100,000 dilution) conjugated with horseradish peroxidase was applied for 1 h at 25 °C. The membranes were developed using the SuperSignal West Femto Maximum sensitivity chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. Membranes were stripped using Restore™ Western Blot Stripping Buffer (Pierce) and reprobed with EphB4 or β-actin.

10) Cord formation assay

Matrigel™ Basement Membrane Matrix (BD Biosciences Discovery Labware, Bedford, MA) was mixed with growth medium (3:1) on ice and 0.5 ml liquid placed in 24-well plates. Incubation of plates at 37 °C for 15 min caused Matrigel™ polymerization. HUVEC or KS-SLK in exponential phase growth were treated with 2 or 8 μg/ml EphB4-ECD or PBS as control for 16 h prior to trypsinizing and plating on the Matrigel™. Culture on Matrigel™ was continued in the presence of recombinant fusion proteins for 6 h. Cultures were fixed in 4% paraformaldehyde for 30 min and evaluated by inverted phase-contrast photomicroscopy.

11) Synthesis of Ephrin B2 and EphB4 siRNA by in vitro transcription

The Silencer™ siRNA construction kit (Ambion, Austin TX) was used to synthesize siRNA to ephrin B2 and EphB4. Briefly, three 21 bp target sequences comprising 19 bp downstream of a 5'-AA dinucleotide were identified in the ephrin B2 cDNA (Accession number NM_004093) that showed no significant homology to other sequences in the GenBank database. Sense and antisense siRNA 29-mer DNA oligonucleotide templates were synthesized at the USC Norris Microchemical Core Facility. Antisense template corresponded to the target sequence followed by 8 bp addition (5'-CCTGTCTC-3') at the 3' end complementary to the T7 promoter primer provided with the Silencer SiRNA Construction Kit. Sense template comprised 5'-AA followed by the complement of the target 19 bp, then the T7 8 bp sequence as above. In separate reactions, the two siRNA oligonucleotide templates were hybridized to a T7 promoter primer. The 3' ends of the hybridized oligonucleotides were extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The dsRNA consisted of 5' terminal single-stranded leader sequences, a 19 nt target specific dsRNA, and 3' terminal UUs. The leader sequences were removed by digesting the dsRNA with a single-stranded specific ribonuclease. The DNA template was removed at the same time by treatment with RNAse free deoxyribonuclease.

The resulting siRNAs were purified by glass fiber filter binding to remove excess nucleotides, short oligomers, proteins, and salts in the reaction. End product double-stranded 21mer siRNAs are shown in Table 5. Similarly, an EphB4 and green fluorescence protein (GFP) siRNAs were synthesized.

Table 5: siRNAs of ephrin B2 and EphB4.

ephrin B2 264	5'-GCAGACAGAUGCACUAUUAUU-3' 3'-UUCGUCUGUCUACGUGAUAAU-5'
ephrin B2 63:	5'-CUGCGAUUUCCAAAUCGAUUU-3' 3'-UUGACGCUAAAGGUUUAGCUA-5'
ephrin B2 137:	5'-GGACUGGUACUAUACCCACUU-3' 3'-UUCCUGACCAUGAUAUGGGUG-5'

Eph B4 50:	5'-GAGACCCUGCUGAACACAAUU-3' 3'-UUCUCUGGGACGACUUGUGUU-5'
GFP	5'-CGCUGACCCUGAAGUUCAUUU-3' 3'-UUGCGACUGGGACUUCAAGUA-5'

12) Transfection of Ephrin B2 or EphB4 siRNA

HUVEC were seeded on eight-well chamber slides coated with fibronectin and grown overnight in EGM-2 (Cambrex, Walkersville, MD). 16 h later media was replaced either with EBM-2 supplemented with 5% fetal calf serum (FCS) and EGM-2 BulletKit supplements bFGF, hEGF and R^3-IGF-I at the concentrations provided by the manufacturer, or EBM-2 supplemented with 5% FCS and 10 ng/ml rhVEGF (R&D Systems). After 2 h incubation at 37 °C, the cells were transfected using Lipofectamine 2000 (1 μg/ml; Invitrogen) and 10 nM specific siRNAs in Opti-MEM-1 serum-free medium (Invitrogen). Following transfection for 2 hr in Opti-MEM-1, media supplemented as above was replaced in the appropriate wells. After 48 hrs, the cells were stained with crystal violet and immediately photographed at 10X magnification.

13) Construction of ephrin B2 reporter plasmids

Human ephrin B2 5'-flanking DNA from -2491 to -11 with respect to the translation start site was amplified from BACPAC clone RP11-297I6 (BacPac Resources, Children's Hospital, Oakland, CA) using the Advantage GC Genomic PCR kit (Clontech Palo Alto, CA) to overcome the large tracts of CG-rich sequence in the target area. Primers were designed to contain *Mlu*I sites for cloning. Amplified product was digested with *Mlu*I, gel purified and ligated into the *Mlu*I site in the multiple cloning site of pGL3Basic (Promega, Madison, WI). Orientation of the resulting clones was confirmed by restriction digest analysis. The correct clone was designated pEFNB2$_{-2491/-11}$luc. Digestion of this clone with either *Kpn*I or *Sac*I followed by recircularization yielded pEFNB2$_{-1242/-11}$luc and pEFNB2$_{-577/-11}$luc, respectively. Plasmid DNAs used for transient transfections were purified using a Mega Prep kit (QIAGEN, Valencia, CA).

14) Transient transfection

HUVEC cells (0.8 x 10^4 cells/well in 24 well plates) maintained in EGM-2 media were transiently co-transfected with 0.5 μg/well ephrin B2 promoter-luciferase constructs together with 50 ng/well either pCEFL or pKSvGPCR-CEFL, using Superfect reagent (QIAGEN) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection and lysed with Luciferase cell lysis buffer (Promega). Luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase was normalized to protein, because pCEFL-vGPCR induced the expression of β-galactosidase from pCMV-Sport-βgal (Invitrogen).

15) Construction and purification of EphB4 extra cellular domain (ECD) protein

See above, e.g., Example 1.

Example 7. Expression of EphB4 in Bladder cancer: a candidate target for therapy

Figure 51 shows expression of EPHB4 in bladder cancer cell lines (A), and regulation of EPHB4 expression by EGFR signaling pathway (B).

Figure 52 shows that transfection of p53 inhibit the expression of EPHB4 in 5637 cell.

Figure 53 shows growth inhibition of bladder cancer cell line (5637) upon treatment with EPHB4 siRNA 472.

Figure 54 shows results on apoptosis study of 5637 cells transfected with EPHB4 siRNA 472.

Figure 55 shows effects of EPHB4 antisense probes on cell migration. 5637 cells were treated with EPHB4AS10 (10 μM).

Figure 56 shows effects of EPHB4 siRNA on cell invasion. 5637 cells were transfected with siRNA 472 or control siRNA.

Example 8. Inhibition of EphB4 Gene Expression by EphB4 antisense probes and RNAi probes

Cell lines expressing EphB4 were treated with the synthetic phosphorothioate modified oligonucleotides and harvested after 24 hr. Cell lysates were prepared and probed by western blot analysis for relative amounts of EphB4 compared to untreated control cells.

Studies on inhibition of cell proliferation were done in HNSCC cell lines characterized to express EphB4. Loss of cell viability was shown upon knock-down of EphB4 expression. Cells were treated in vitro and cultured in 48-well plates, seeded with 10 thousand cells per well. Test compounds were added and the cell viability was tested on day 3. The results on EphB4 antisense probes were summarized below in Table 6. The results on EphB4 RNAi probes were summarized below in Table 7.

Table 6. Inhibition of EphB4 Gene Expression by EphB4 antisense probes

Name	Sequence 5'→ 3'	position	Inhibition of EphB4 Expression	Percent reduction in viability
Eph B4 169	TCA GTA CTG CGG GGC CGG TCC	(2944-2963)	++	36
Eph B4 168	TCC TGT CCC ACC CGG GGT TC	(2924-2943)	++	51
Eph B4 167	CCG GCT TGG CCT GGG ACT TC	(2904-2923)	+++	66
Eph B4 166	ATG TGC TGG ACA CTG GCC AA	(2884-2903)	++++	70
Eph B4 165	GAT TTT CTT CTG GTG TCC CG	(2864-2883)	++++	75
Eph B4 164	CCA GAG TGA CTC CGA TTC GG	(2844-2863)	++	40
Eph B4 163	AGC AGG TCC TCA GCA GAG AT	(2824-2843)	++++	66
Eph B4 162	CTG GCT GAC CAG CTC GAA GG	(2804-2823)		25
Eph B4 161	AGC CAA AGC CAG CGG CTG CG	(2784-2803)	+	33
Eph B4 160	AAA CTT TCT TCG TAT CTT CC	(2763-2783)	+	25
Eph B4 159	CAT TTT GAT GGC CCG AAG CC	(2743-2762)	++	40
Eph B4 158	ACT CGC CCA CAG AGC CAA AA	(2723-2742)		30
Eph B4 157	GCT GAG TAG TGA GGC TGC CG	(2703-2722)	+	25
Eph B4 156	CTG GTC CAG GAG AGG GTG TG	(2683-2702)	++	30
Eph B4 155	AGG CCC CGC CAT TCT CCC GG	(2663-2682)		25
Eph B4 154	GCC ACG ATT TTG AGG CTG GC	(2643-2662)	++	40
Eph B4 153	GGG GTT CCG GAT CAT CTT GT	(2623-2642)	++	35
Eph B4 152	CCA GGG CGC TGA CCA CCT GG	(2603-2622)	+	30
Eph B4 151	GGG AAG CGG GGC CGG GCA TT	(2583-2602)	+	25
Eph B4 150	CCG GTC TTT CTG CCA ACA GT	(2563-2582)	++	25
Eph B4 149	CCA GCA TGA GCT GGT GGA GG	(2543-2562)	++	20
Eph B4 148	GAG GTG GGA CAG TCT GGG GG	(2523-2542)	+	30
Eph B4 147	CGG GGG CAG CCG GTA GTC CT	(2503-2522)	++	40
Eph B4 146	GTT CAA TGG CAT TGA TCA CG	(2483-2502)	++++	70
Eph B4 145	TCC TGA TTG CTC ATG TCC CA	(2463-2482)	++++	80
Eph B4 144	GTA CGG CCT CTC CCC AAA TG	(2443-2462)	+++	60
Eph B4 143	ACA TCA CCT CCC ACA TCA CA	(2423-2442)	++++	80
Eph B4 142	ATC CCG TAA CTC CAG GCA TC	(2403-2422)	++	40
Eph B4 141	ACT GGC GGA AGT GAA CTT CC	(2383-2402)	+++	50
Eph B4 140	GGA AGG CAA TGG CCT CCG GG	(2363-2382)	++	45
Eph B4 139	GCA GTC CAT CGG ATG GGA AT	(2343-2362)	++++	70
Eph B4 138	CTT TCC TCC CAG GGA GCT CG	(2323-2342)	++++	70
Eph B4 137	TGT AGG TGG GAT CGG AAG AG	(2303-2322)	++	40
Eph B4 136	TTC TCC TCC AGG AAT CGG GA	(2283-2302)	++	35
Eph B4 135	AAG GCC AAA GTC AGA CAC TT	(2263-2282)	++++	60
Eph B4 134	GCA GAC GAG GTT GCT GTT GA	(2243-2262)	++	50

Eph B4 133	CTA GGA TGT TGC GAG CAG CC	(2223-2242)	++	40
Eph B4 132	AGG TCT CGG TGG ACG TAG CT	(2203-2222)	++	40
Eph B4 131	CAT CTC GGC AAG GTA CCG CA	(2183-2202)	+++	50
Eph B4 130	TGC CCG AGG CGA TGC CCC GC	(2163-2182)	++	50
Eph B4 129	AGC ATG CCC ACG AGC TGG AT	(2143-2162)	++	50
Eph B4 128	GAC TGT GAA CTG TCC GTC GT	(2123-2142)	++	50
Eph B4 127	TTA GCC GCA GGA AGG AGT CC	(2103-2122)	+++	60
Eph B4 126	AGG GCG CCG TTC TCC ATG AA	(2083-2102)	++	50
Eph B4 125	CTC TGT GAG AAT CAT GAC GG	(2063-2082)	++++	80
Eph B4 124	GCA TGC TGT TGG TGA CCA CG	(2043-2062)	++++	70
Eph B4 123	CCC TCC AGG CGG ATG ATA TT	(2023-2042)	++	50
Eph B4 122	GGG GTG CTC GAA CTG GCC CA	(2003-2022)	++++	80
Eph B4 121	TGA TGG AGG CCT CGC TCA GA	(1983-2002)	++	50
Eph B4 120	AAC TCA CGC CGC TGC CGC TC	(1963-1982)	++	40
Eph B4 119	CGT GTA GCC ACC CTT CAG GG	(1943-1962)	++++	75
Eph B4 118	TCT TGA TTG CCA CAC AGC TC	(1923-1942)	++++	80
Eph B4 117	TCC TTC TTC CCT GGG GCC TT	(1903-1922)	++++	70
Eph B4 116	GAG CCG CCC CCG GCA CAC CT	(1883-1902)	++	50
Eph B4 115	CGC CAA ACT CAC CTG CAC CA	(1863-1882)	++++	60
Eph B4 114	ATC ACC TCT TCA ATC TTG AC	(1843-1862)	++++	65
Eph B4 113	GTA GGA GAC ATC GAT CTC TT	(1823-1842)	++++	90
Eph B4 112	TTG CAA ATT CCC TCA CAG CC	(1803-1822)	++++	70
Eph B4 111	TCA TTA GGG TCT TCA TAA GT	(1783-1802)	++++	70
Eph B4 110	GAA GGG GTC GAT GTA GAC CT	(1763-1782)	++++	80
Eph B4 109	TAG TAC CAT GTC CGA TGA GA	(1743-1762)	++	50
Eph B4 108	TAC TGT CCG TGT TTG TCC GA	(1723-1742)	++	45
Eph B4 107	ATA TTC TGC TTC TCT CCC AT	(1703-1722)	++++	70
Eph B4 106	TGC TCT GCT TCC TGA GGC AG	(1683-1702)	++++	70
Eph B4 105	AGA ACT GCG ACC ACA ATG AC	(1663-1682)	++	40
Eph B4 104	CAC CAG GAC CAG GAC CAC AC	(1643-1662)	++++	70
Eph B4 103	CCA CGA CTG CCG TGC CCG CA	(1623-1642)	++	40
Eph B4 102	ATC AGG GCC AGC TGC TCC CG	(1603-1622)	+++	50
Eph B4 101	CCA GCC CTC GCT CTC ATC CA	(1583-1602)	++++	80
Eph B4 100	GTT GGG TCT GGC TGT GAT GT	(1563-1582)	++++	80
Eph B4 99	TCC TGG CCG AAG GGC CCG TA	(1543-1562)	++	35
Eph B4 98	GCC GGC CTC AGA GCG CGC CC	(1523-1542)	++	50
Eph B4 97	GTA CCT GCA CCA GGT AGC TG	(1503-1522)	++++	80
Eph B4 96	GCT CCC CGC TTC AGC CCC CG	(1483-1502)	++	50
Eph B4 95	CAG CTC TGC CCG GTT TTC TG	(1463-1482)	++	50
Eph B4 94	ACG TCT TCA GGA ACC GCA CG	(1443-1462)	++++	80
Eph B4 93	CTG CTG GGA CCC TCG GCG CC	(1423-1442)	++	40
Eph B4 92	CTT CTC ATG GTA TTT GAC CT	(1403-1422)	++++	80
Eph B4 91	CGT AGT CCA GCA CAG CCC CA	(1383-1402)	++++	85
Eph B4 90	CTG GGT GCC CGG GGA ACA GC	(1363-1382)	+++	50
Eph B4 89	CCA GGC CAG GCT CAA GCT GC	(1343-1462)	++++	70
Eph B4 88	TGG GTG AGG ACC GCG TCA CC	(1323-1342)	++	40
Eph B4 87	CGG ATG TCA GAC ACT GCA GG	(1303-1322)	++++	60
Eph B4 86	AGG TAC CTC TCG GTC AGT GG	(1283-1302)	++	50
Eph B4 85	TGA CAT TGA CAG GCT CAA AT	(1263-1282)	++++	80
Eph B4 84	GGG ACG GGC CCC GTG GCT AA	(1243-1262)	++	50
Eph B4 83	GGA GGA TAC CCC GTT CAA TG	(1223-1242)	+++	60
Eph B4 82	CAG TGA CCT CAA AGG TAT AG	(1203-1222)	++++	70
Eph B4 81	GTG AAG TCA GGA CGT AGC CC	(1183-1202)	+++	60
Eph B4 80	TCG AAC CAC CAC CCA GGG CT	(1163-1182)	+++	50

Eph B4 79	CCA CCA GGT CCC GGG GGC CG	(1143-1162)	++	40
Eph B4 78	GGG TCA AAA GTC AGG TCT CC	(1123-1142)	++++	70
Eph B4 77	CCC GCA GGG CGC ACA GGA GC	(1103-1122)	+++	60
Eph B4 76	CTC CGG GTC GGC ACT CCC GG	(1083-1102)	+++	60
Eph B4 75	CAG CGG AGG GCG TAG GTG AG	(1063-1082)	++	40
Eph B4 74	GTC CTC TCG GCC ACC AGA CT	(1043-1062)	++	50
Eph B4 73	CCA GGG GGG CAC TCC ATT CC	(1023-1042)	++	50
Eph B4 72	AGG TGC AGG GAG GAG CCG TT	(1003-1022)	++++	70
Eph B4 71	CAG GCG GGA AAC CAC GCT CC	(983-1002)	++	40
Eph B4 70	GCG GAG CCG AAG GAG GGG TG	(963-982)	+++	50
Eph B4 69	GTG CAG GGT GCA CCC CGG GG	(943-962)	+++	50
Eph B4 68	GTC TGT GCG TGC CCG GAA GT	(923-942)	++	40
Eph B4 67	ACC CGA CGC GGC ACT GGC AG	(903-922)	++	40
Eph B4 66	ACG GCT GAT CCA ATG GTG TT	(883-902)	++	50
Eph B4 65	AGA GTG GCT ATT GGC TGG GC	(863-882(	++++	60
Eph B4 64	ATG GCT GGC AGG ACC CTT CT	(843-862)	++++	80
Eph B4 63	CCT GAC AGG GGC TTG AAG GT	(823-842)	++++	80
Eph B4 62	GCC CTG GGC ACA GGC TCG GC	(803-822)	+++	70
Eph B4 61	ACT TGG TGT TCC CCT CAG CT	(783-802)	++++	80
Eph B4 60	GCC TCG AAC CCC GGA GCA CA	(763-782)	+++	50
Eph B4 59	GCT GCA GCC CGT GAC CGG CT	(743-762)	+++	50
Eph B4 58	GTT CGG CCC ACT GGC CAT CC	(723-742)	++	45
Eph B4 57	TCA CGG CAG TAG AGG CTG GG	(703-722)	+++	70
Eph B4 56	GCT GGG GCC AGG GGC GGG GA	(683-702)	++	50
Eph B4 55	CGG CAT CCA CCA CGC AGC TA	(663-682)	++	50
Eph B4 54	CCG GCC ACG GGC ACA ACC AG	(643-662)	++	50
Eph B4 53	CTC CCG AGG CAC AGT CTC CG	(623-642)	+++	50
Eph B4 52	GGA ATC GAG TCA GGT TCA CA	(603-622)	++++	90
Eph B4 51	GTC AGC TGG GCG CAC TTT TT	(583-602)	+++	70
Eph B4 50	GTA GAA GAG GTG CAG GGA TA	(563-582)	++++	80
Eph B4 49	GCA GGG CCA TGC AGG CAC CC	(543-562)	++++	80
Eph B4 48	TGG TCC TGG AAG GCC AGG TA	(523-542)	++++	90
Eph B4 47	GAA GCC AGC CTT GCT GAG CG	(503-522)	++++	80
Eph B4 46	GTC CCA GAC GCA GCG TCT TG	(483-502)	++	40
Eph B4 45	ACA TTC ACC TTC CCG GTG GC	(463-482)	+++	50
Eph B4 44	CTC GGC CCC AGG GCG CTT CC	(443-462)	++	50
Eph B4 43	GGG TGA GAT GCT CCG CGG CC	(423-442)	+++	60
Eph B4 42	ACC GTG TCC ACC TTG ATG TA	(403-422)	++++	80
Eph B4 41	GGG GTT CTC CAT CCA GGC TG	(383-402)	++++	80
Eph B4 40	GCG TGA GGG CCG TGG CCG TG	(363-382)	++	50
Eph B4 39	TCC GCA TCG CTC TCA TAG TA	(343-362)	+++	60
Eph B4 38	GAA GAC GGT GAA GGT CTC CT	(323-342)	++++	80
Eph B4 37	TGC AGG AGC GCC CAG CCC GA	(303-322)	+++	50
Eph B4 36	GGC AGG GAC AGG CAC TCG AG	(283-302)	+++	45
Eph B4 35	CAT GGT GAA GCG CAG CGT GG	(263-282)	++	50
Eph B4 34	CGT ACA CGT GGA CGG CGC CC	(243-262)	++	40
Eph B4 33	CGC CGT GGG ACC CAA CCT GT	(223-242)	+++	60
Eph B4 32	GCG AAG CCA GTG GGC CTG GC	(203-222)	++++	70
Eph B4 31	CCG GGG CAC GCT GCA CGT CA	(183-202)	+++	60
Eph B4 30	CAC ACT TCG TAG GTG CGC AC	(163-182)	+++	70
Eph B4 29	GCT GTG CTG TTC CTC ATC CA	(143-162)	++++	80
Eph B4 28	GGC CGC TCA GTT CCT CCC AC	(123-142)	++	40
Eph B4 27	TGC CCG TCC ACC TGA GGG AA	(103-122)	++	50
Eph B4 26	TGT CAC CCA CTT CAG ATC AG	(83-102)	++++	70

Eph B4 25	CAG TTT CCA ATT TTG TGT TC	(63-82)	++++	70
Eph B4 24	AGC AGG GTC TCT TCC AAA GC	(43-62)	++++	80
Eph B4 23	TGC GGC CAA CGA AGC CCA GC	(23-42)	++	50
Eph B4 22	AGA GCA GCA CCC GGA GCT CC	(3-22)	+++	50
Eph B4 21	AGC AGC ACC CGG AGC TCC AT	(1-20)	+++	50
Additional antisense probes described in the specification				
EphB4 AS-1	GTG CAG GGA TAG CAG GGC CAT	(552-572)		
EphB4 AS-2	AAG GAG GGG TGG TGC ACG GTG	(952-972)		
EphB4 AS-3	TTC CAG GTG CAG GGA GGA GCC	(1007-1027)		
EphB4 AS-4	GTG GTG ACA TTG ACA GGC TCA	(1263-1285)		
EphB4 AS-5	TCT GGC TGT GAT GTT CCT GGC	(1555-1575)		
EphB4 AS-6	GCC GCT CAG TTC CTC CCA	(123-140)		
EphB4 AS-7	TGA AGG TCT CCT TGC AGG	(316-333)		
EphB4 AS-8	CGC GGC CAC CGT GTC CAC CTT	(408-428)		
EphB4 AS-9	CTT CAG GGT CTT GAT TGC CAC	(1929-1949)		
EphB4 AS-10	ATG GAG GCC TCG CTC AGA AA	(1980-1999)		
Ephb4 AS-11	CAT GCC CAC GAG CTG GAT GAC	(2138-2158)		

Table 7. Inhibition of EphB4 Gene Expression by EphB4 RNAi probes

RNAi	EphB4 RNAi sequence			Inhibition of EphB4 Expression	Percent reduction in viability
1	446	aaattggaaactgctgatctg	466		
2	447	aattggaaactgctgatctga	467	+++	70
3	453	aaactgctgatctgaagtggg	473	++++	70
4	454	aactgctgatctgaagtgggt	474	+++	80
5	854	aatgtcaagacgctgcgtctg	874	+++	65
6	467	aagtgggtgacattccctcag	487	+	35
7	848	aaggtgaatgtcaagacgctg	868	++	50
8	698	aaggagaccttcaccgtcttc	718	+++	75
9	959	aaaaagtgcgcccagctgact	979	+	40
10	1247	aatagccactctaacaccatt	1267	++	50
11	1259	aacaccattggatcagccgtc	1279	++	50
12	1652	aatgtcaccactgaccgagag	1672	+	35
13	1784	aaataccatgagaagggcgcc	1804	+++	65
14	1832	aagacgtcagaaaaccgggca	1852	+	30
15	1938	aacatcacagccagacccaac	19	++	50
16	2069	aagcagagcaatgggagagaa	2089	++++	75
17	2078	aatgggagagaagcagaatat	2098	+++	65
18	2088	aagcagaatattcggacaaac	2108	+++	70

19	2094	aatattcggacaaacacggac	2114	++	40
20	2105	aaacacggacagtatctcatc	2125	++	50
21	2106	aacacggacagtatctcatcg	2126	+	35
22	2197	aaaagagatcgatgtctccta	2217	+++	65
23	2174	aatgaggctgtgagggaattt	2194	++	50
24	2166	aagaccctaatgaggctgtga	2186	++	50
25	2198	aaagagatcgatgtctcctac	2218	+++	55
26	2199	aagagatcgatgtctcctacg	2219	+++	70
27	2229	aagaggtgattggtgcaggtg	2249	+	33
28	2222	aagattgaagaggtgattggt	2242	+	30
29	2429	aacagcatgcccgtcatgatt	2449	++	40
30	2291	aagaaggagagctgtgtggca	2311	+++	50
31	2294	aaggagagctgtgtggcaatc	2314	+++	60
32	2311	aatcaagaccctgaagggtgg	2331	+++	70
33	2497	aaacgacggacagttcacagt	2517	+	35
34	2498	aacgacggacagttcacagtc	2518	+	40
35	2609	aacatcctagtcaacagcaac	2629	++	50
36	2621	aacagcaacctcgtctgcaaa	2641	+	35
37	2678	aactcttccgatcccacctac	2698	++	50
38	2640	aagtgtctgactttggccttt	2660	+++	70
39	2627	aacctcgtctgcaaagtgtct	2647	++	50
40	2639	aaagtgtctgactttggcctt	2659	+	25
41	2852	aatcaggacgtgatcaatgcc	2872	+++	75
42	2716	aaagattcccatccgatggac	2736	++	50
43	2717	aagattcccatccgatggact	2737	++	60
44	2762	aagttcacttccgccagtgat	2782	+++	70
45	3142	aagatacgaagaaagtttcgc	3162	++	50
46	3136	aatgggaagatacgaagaaag	3156	+++	66
47	2867	aatgccattgaacaggactac	2887		
48	3029	aaaatcgtggcccgggagaat	3049	+	33
49	3254	aaaatcttggccagtgtccag	3274	++	50
50	3255	aaatcttggccagtgtccagc	3275	+++	75
51	3150	aagaaagtttcgcagccgctg	3170	+++	80
52	3251	aagaaaatcttggccagtgtc	3271	++	50
53	3256	aatcttggccagtgtccagca	3276	++	50
Additional RNAi probes described in the specification					
Eph B4 50		gagacccugcugaacacaauu			
Eph B4 472		ggugaaugucaagacgcuguu			
Eph B4 1562		caucacagccagacccaacuu			

siRNA 2303	cucuuccgaucccaccuacuu		
Eph B4 2302	cucuuccgaucccaccuacuu		

Example 9. Inhibition of Ephrin B2 Gene Expression by Ephrin B2 antisense probes and RNAi probes

KS SLK, a cell line expressing endogenous high level of ephrin B2. Cell viability was tested using fixed dose of each oligonuceotide (5UM). Gene expression downregulation was done using cell line 293 engineered to stably express full-length ephrin B2. KS SLK expressing EphrinB2 were also used to test the viability in response to RNAi probes tested at the fixed dose of 50 nM. Protein expression levels were measured using 293 cells stably expressing full-length EphrinB2, in cell lysates after 24 hr treatment with fixed 50 nM of RNAi probes.

The results on Ephrin B2 antisense probes were summarized below in Table 8. The results on Ephrin B2 RNAi probes were summarized below in Table 9.

Table 8. Ephrin B2 antisense ODNs.

	sequence	Coding region	Percent reduction in viability	Inhibition of Ephrin B2 Expression
Ephrin AS-51	TCA GAC CTT GTA GTA AAT GT	(983-1002)	35	++
Ephrin AS-50	TCG CCG GGC TCT GCG GGG GC	(963-982)	50	+++
Ephrin AS-49	ATC TCC TGG ACG ATG TAC AC	(943-962)	45	++
Ephrin AS-48	CGG GTG CCC GTA GTC CCC GC	(923-942)	35	++
Ephrin AS-47	TGA CCT TCT CGT AGT GAG GG	(903-922)	40	+++
Ephrin AS-46	CAG AAG ACG CTG TCC GCA GT	(883-902)	40	++
Ephrin AS-45	CCT TAG CGG GAT GAT AAT GT	(863-882)	35	++
Ephrin AS-44	CAC TGG GCT CTG AGC CGT TG	(843-862)	60	+++
Ephrin AS-43	TTG TTG CCG CTG CGC TTG GG	(823-842)	40	++
Ephrin AS-42	TGT GGC CAG TGT GCT GAG CG	(803-822)	40	++
Ephrin AS-41	ACA GCG TGG TCG TGT GCT GC	(783-802)	70	+++
Ephrin AS-40	GGC GAG TGC TTC CTG TGT CT	(763-782)	80	++++
Ephrin AS-39	CCT CCG GTA CTT CAG CAA GA	(743-762)	50	+++
Ephrin AS-38	GGA CCA CCA GCG TGA TGA TG	(723-742)	60	+++
Ephrin AS-37	ATG ACG ATG AAG ATG ATG CA	(703-722)	70	+++
Ephrin AS-36	TCC TGA AGC AAT CCC TGC AA	(683-702)	60	+++
Ephrin AS-35	ATA AGG CCA CTT CGG AAC CG	(663-682)	45	++
Ephrin AS-34	AGG ATG TTG TTC CCC GAA TG	(643-662)	50	+++
Ephrin AS-33	TCC GGC GCT GTT GCC GTC TG	(623-642)	75	+++
Ephrin AS-32	TGC TAG AAC CTG GAT TTG GT	(603-622)	60	+++
Ephrin AS-31	TTT ACA AAG GGA CTT GTT GT	(583-602)	66	+++
Ephrin AS-30	CGA ACT TCT TCC ATT TGT AC	(563-582)	50	++
Ephrin AS-29	CAG CTT CTA GTT CTG GAC GT	(543-562)	50	+++
Ephrin AS-28	CTT GTT GGA TCT TTA TTC CT	(523-542)	70	+++
Ephrin AS-27	GGT TGA TCC AGC AGA ACT TG	(503-522)	65	+++
Ephrin AS-26	CAT CTT GTC CAA CTT TCA TG	(483-502)	75	+++
Ephrin AS-25	AGG ATC TTC ATG GCT CTT GT	(463-482)	60	+++
Ephrin AS-24	CTG GCA CAC CCC TCC CTC CT	(443-462)	45	++

Ephrin AS-23	GGT TAT CCA GGC CCT CCA AA	(423-442)	50	+++
Ephrin AS-22	GAC CCA TTT GAT GTA GAT AT	(403-422)	50	+++
Ephrin AS-21	AAT GTA ATA ATC TTT GTT CT	(383-402)	60	+++
Ephrin AS-20	TCT GAA ATT CTA GAC CCC AG	(363-382)	60	+++
Ephrin AS-19	AGG TTA GGG CTG AAT TCT TG	(343-362)	75	+++
Ephrin AS-18	AAA CTT GAT GGT GAA TTT GA	(323-342)	60	+++
Ephrin AS-17	TAT CTT GGT CTG GTT TGG CA	(303-322)	50	++
Ephrin AS-16	CAG TTG AGG AGA GGG GTA TT	(283-302)	40	++
Ephrin AS-15	TTC CTT CTT AAT AGT GCA TC	(263-282)	66	+++
Ephrin AS-14	TGT CTG CTT GGT CTT TAT CA	(243-262)	70	++++
Ephrin AS-13	ACC ATA TAA ACT TTA TAA TA	(223-242)	50	+++
Ephrin AS-12	TTC ATA CTG GCC AAC AGT TT	(203-222)	50	+++
Ephrin AS-11	TAG AGT CCA CTT TGG GGC AA	(183-202)	70	++++
Ephrin AS-10	ATA ATA TCC AAT TTG TCT CC	(163-182)	70	++++
Ephrin AS-9	TAT CTG TGG GTA TAG TAC CA	(143-162)	80	++++
Ephrin AS-8	GTC CTT GTC CAG GTA GAA AT	(123-142)	60	+++
Ephrin AS-7	TTG GAG TTC GAG GAA TTC CA	(103-122)	80	++++
Ephrin AS-6	ATA GAT AGG CTC TAA AAC TA	(83-102)	70	+++
Ephrin AS-5	TCG ATT TGG AAA TCG CAG TT	(63-82)	50	+++
Ephrin AS-4	CTG CAT AAA ACC ATC AAA AC	(43-62)	80	++++
Ephrin AS-3	ACC CCA GCA GTA CTT CCA CA	(23-42)	85	++++
Ephrin AS-2	CGG AGT CCC TTC TCA CAG CC	(3-22)	70	+++
Ephrin AS-1	GAG TCC CTT CTC ACA GCC AT	(1-20)	80	++++

Table 9. Ephrin B2 RNAi probes.

RNAi Sequence and homology with other human genes.			Percent reduction in viability	Inhibition of Ephrin B2 Expression	RNAi no.
89	aactgcgatttccaaatcgat	109	80	++++	1
141	aactccaaatttctacctgga	161	70	++++	2
148	aatttctacctggacaaggac	168	75	+++	3
147	aaatttctacctggacaagga	167	60	+++	4
163	aaggactggtactatacccac	183	40	++	5
217	aagtggactctaaaactgttg	237	80	++++	6
229	aaactgttggccagtatgaat	249	50	+++	7
228	aaaactgttggccagtatgaa	248	80	++++	8
274	aagaccaagcagacagatgca	294	80	++++	11
273	aaagaccaagcagacagatgc	293	60	+++	12
363	aagtttcaagaattcagccct	383	66	+++	13
370	aagaattcagccctaacctct	390	50	+++	14
373	aattcagccctaacctctggg	393	50	+++	15
324	aactgtgccaaaccagaccaa	344	90	++++	16
440	aaatgggtctttggagggcct	460	80	++++	17
501	aagatcctcatgaaagttgga	521	50	+++	18
513	aaagttggacaagatgcaagt	533	50	+++	19
491	aagagccatgaagatcctcat	511	50	+++	20

514	aagttggacaagatgcaagtt	534	66	+++	21
523	aagatgcaagttctgctggat	543	66	+++	22
530	aagttctgctggatcaaccag	550	50	+++	23
545	aaccaggaataaagatccaac	565	35	++	24
555	aaagatccaacaagacgtcca	575	40	++	25
556	aagatccaacaagacgtccag	576	60	+++	26
563	aacaagacgtccagaactaga	583	60	+++	27
566	aagacgtccagaactagaagc	586	70	+++	28
593	aaatggaagaagttcgacaac	613	75	++++	29
577	aactagaagctggtacaaatg	597	66	+++	30
594	aatggaagaagttcgacaaca	614	35	++	31
583	aagctggtacaaatggaagaa	603	50	+++	32
611	aacaagtccctttgtaaaacc	631	70	++++	33
599	aagaagttcgacaacaagtcc	619	70	++++	34
602	aagttcgacaacaagtccctt	622	80	++++	35
626	aaaaccaaatccaggttctag	646	50	+++	36
627	aaaccaaatccaggttctagc	647	25	+	37
628	aaccaaatccaggttctagca	648	30	++	38
632	aaatccaggttctagcacaga	652	60	+++	39
633	aatccaggttctagcacagac	653	40	++	40
678	aacaacatcctcggttccgaa	698	30	++	41
681	aacatcctcggttccgaagtg	701	20	+	42
697	aagtggccttatttgcaggga	717	30	++	43
Additional Ephrin B2 RNAi probes described in the specification					
GCAGACAGAUGCACUAUUAUU					ephrin B2 264
CUGCGAUUUCCAAAUCGAUUU					ephrin B2 63
GGACUGGUACUAUACCCACUU					ephrin B2 137

Example 10. EphB4 antibodies inhibit tumor growth

Figure 57 shows results on comparison of EphB4 monoclonal antibodies by G250 and in Pull-down assay.

Figure 58 shows that EphB4 antibodies, in the presence of matrigel and growth factors, inhibit the *in vivo* tumor growth of SCC15 cells.

BalbC nude mice were injected subcutaneously with 2.5 x 10^6 viable tumor cells SCC15 is a head and neck squamous cell carcinoma line. Tumors were initiated in nu/nu mice by injecting 2.5-5x10^6 cells premixed with matrigel and Growth factors, and Ab's subcutaneously to initiate tumor xenografts. Mice were opened 14 days after injections. SCC15 is a head and neck squamous cell carcinoma line, B16 is a melanoma cell line, and MCF-7 is a breast carcinoma line. The responses of tumors to these treatments were compared to control treated mice, which receive PBS injections. Animals were observed daily for tumor growth and subcutaneous tumors were measured using a caliper every 2 days. Antibodies #1 and #23 showed significant regression of SCC15 tumor size compared to control, especially with no additional growth factor added.

Figure 59 shows that EphB4 antibodies cause apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

Angiogenesis was assessed by CD-31 immunohistochemistry. Tumor tissue sections from treated and untreated mice were stained for CD31. Apoptosis was assessed by immunohistochemical TUNNEL, and proliferation by BrdU assay. Following surgical removal, tumors were immediately sliced into 2 mm serial sections and embedded in paraffin using standard procedures. Paraffin embedded tissue were sectioned at 5 μm, the wax removed and the tissue rehydrated. The rehydrated tissues were microwave irradiated in antigen retreival solution. . Slides were rinsed in PBS, and TUNNEL reaction mixture (Terminal deoxynucleotidyl transferase and flourescein labeled nucleotide solution), and BrdU were added in a humidity chamber completely shielded from light. The TUNNEL and BrdU reaction mixture were then removed, slides were rinsed and anti-flourescein antibody conjugated with horseradish peroxidase was added. After incubation and rinsing, 3, 3'diaminobenzidine was added. Masson's Trichrome and Hematoxylin and Eosin were also used to stain the slides to visualize morphology. Masson's Trichrome allows to visualize necrosis and fibrosis. The tumor gets blood support from tumor/skin, muscle boundary. As tumor grows, inner regions get depleted of nutrients. This leads to necrosis (cell death), preferably at the tumor center. After cells die, (tumor) tissue gets replaced with fibroblastic tissue. Slides were visualized under 20-fold magnification with digital images acquired. A different morphology was obtained on SCC tumors with each antibody administered. Ab #1 showed an increase in necrosis and fibrosis but

not apoptosis. Ab #23 showed an increase in apoptosis, necrosis and fibrosis and a decrease in vessel infiltration. Ab #35 showed an increase in necrosis and fibrosis, and a small increase in apoptosis and a decrease in vessel infiltration. Ab #79 showed a large increase in apoptosis, and necrossis and fibrosis. Ab #91 showed no change in apoptosis but an increase in proliferation. And Ab #138 showed an increase in apoptosis, necrosis, fibrosis and a decrease in proliferation and vessel infiltration. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic response. Tumors treated with EphB4 antibodies displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 60 shows that systemic administration of antibodies on xenografts leads to tumor regression in SCC15 tumor xenografts.

Alternate day treatment with EphB4 monoclonal antibody or an equal volume of PBS as control were initiated on day 4, after the tumors have established, and continued for 14 days. Systemic administration was administered either IP or SC with no significant difference. All the experiments were carried out in a double-blind manner to eliminate investigator bias. Mice were sacrificed at the conclusion of the two week treatment period. Tumors were harvested immediately postmortem and fixed and processed for immunohistochemistry. EphB4 antibodies 40 mg per kg body weight were administered. Treatment with EphB4 antibody significantly inhibited human SCC tumor growth compared with control-treated mice ($p<0.05$). Treatment with EphB4 antibody significantly inhibited tumor weight compared with control-treated mice ($p<0.05$).

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become

apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

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